REMARKS

The specification has been amended at page 1 to include a paragraph reciting the claim of benefit of the earlier related applications.

Claim 2 has been amended to recite an immunogenic composition, to match the body of the claim to the preamble.

Claim 3 has been amended to correct a typographical error and to insert the alternative designation used in the specification. These amendments to claim 3 do not affect the scope of the claim.

THE INVENTION

The claimed invention relates to a method for the treatment of gastrointestinal tumors that are dependent on glycine-extended gastrin-17 (hereinafter "G17Gly"), the method includes administering a therapeutically effective amount of an anti-G17 immunogenic composition to a mammal.

The anti-G17 immunogenic composition elicits an antibody that binds G17Gly and prevents its stimulatory action on the G17Gly-dependent tumor.

I. Rejection Under 35 U.S.C. § 112, Second Paragraph

In the Office Action of November 1st, 2005, claim 2 was rejected under 35 U.S.C. § 112, second paragraph as allegedly unclear in the recitation of "immunogen" in claim 2. According to the Office Action, this term lacks an antecedent basis in claim 1, which recites an anti-G17 "immunogenic composition."

Applicant has herein amended claim 2 to recite an anti-G17 "immunogenic composition." Therefore, the rejection of claim 2 under 35 U.S.C. § 112, second paragraph should be withdrawn.

II. Rejection Under 35 U.S.C. § 112 first paragraph

In the Office Action of November 1, 2005, claims 1-5 were rejected under 35 U.S.C. § 112 first paragraph as allegedly failing to comply with the enablement requirement. According to the Patent and Trademark Office, the specification fails to describe the subject matter of claims 1-5 in such a way as to enable one of ordinary skill in the art to practice the invention.

The Office Action advances the argument that the specification does not disclose an example of a G17Gly-dependent tumor, and that further, the concept of glycine-extended gastrin as a growth factor was controversial at the time of the invention.

These arguments are erroneous: DHDK12 is a G17Gly-dependent tumor cell line, as amply demonstrated by the Examples in the present specification. Applicants respectfully point out that, contrary to the Examiner's assertion, the specification is replete with evidence from *in vitro* and *in vivo* experiments of the dependence of DHDK12 tumor cells on G17Gly and treatment of such tumors by passive immunization with an anti-G17 antibody (Example 2, page 14, Table 2), and by active immunization with a G17:DT immunogen (Example 3, pages 16-17 and Figures 4 and 5) as detailed below.

The specification at page 13, Table 1 shows that in two separate *in vitro* cell culture experiments, DHDK12 cells produced 30-80 fmol G17Gly per 10^7 cells, but no detectable amidated G17. Further, as stated above, the G17Gly produced by the DHDK12 cells was reduced to undetectable levels by treatment with rabbit antibody raised against G17:DT. That this effect is due to the anti-G17 antibodies and not the anti-DT component is shown by the addition of rabbit anti-DT antibody as control, which did not affect the level of G17Gly, measured as 67 fmol per 10^7 cells in this experiment (See Example 2,

Table 2 at page 14). These experiments show that antibody raised against G17:DT neutralizes the G17Gly produced by the DHDK12 cells.

Further, Experiment 3, pages 15-17, demonstrates that anti-G17 antibody elicited by immunization with G17:DT markedly inhibits the growth of syngeneic rat DHDK12 tumors *in vivo*. The ablation of G17 and reduction of G17Gly *in vivo* resulted in the reduction in tumor size (70.2% reduction in tumor cross-sectional area as compared with untreated controls, Example 3, Figure 4 and paragraph spanning pages 16-17) and tumor weight (56.5% reduction in weight as compared to tumors from the DT-treated controls, Example 3, Figure 5 and page 17 first paragraph).

Thus, the specification provides detailed evidence of the existence of G17Gly-dependent tumors, showing that the DHDK12 cell line is G17Gly-dependent and that intervention with an anti-G17 immunogenic composition elicits an antibody that binds G17Gly and prevents its stimulatory action on the G17Gly-dependent tumor.

The existence of G17Gly-dependent tumors is recognized not only in the present specification, but also in the art at the time the invention was filed. The publication of Seva et al. (1994 Science 265: 410-412, cited in the specification at page 3, second complete paragraph), clearly teaches the role of G17Gly in the stimulation of the tumor-derived pancreatic cell line, AR4-2J, and demonstrates that the G17Gly acts through a mechanism independent of the gastrin/CCK-B receptors. Seva et al. implicated these receptors for G17Gly as mediators of the physiological and pathophysiological effects of G17Gly on G17Gly-dependent tumors (Seva, see the title: "Growth-Promoting Effects of Glycine-Extended Progastrin" and page 412 col. 1, first sentence, last paragraph). A copy of the Seva et al publication is attached for the Examiner's convenience.

The existence of G17Gly-dependent tumors was known in the art prior to the present invention and confirmed by the Applicants as shown by the data presented in the present specification. G17Gly-dependent tumors are now well recognized in the art. See for example, Koh et al., (2004) Glycine-Extended Gastrin Promotes the Growth of Lung Cancer, Cancer Research 64: 196-201 (Copy attached).

Moreover, the specification clearly teaches how to recognize a G17Gly-dependent tumor, contrary to the assertions in the Office Action of November 1, 2005. Just as for DHDK12 tumor cells, other tumor cell candidates can readily be assessed for G17Gly-dependence by either passively or actively immunizing to provide anti-G17 antibodies and assessing G17 and G17Gly levels and the effects of reducing or removing G17Gly activity under conditions where G17 is absent.

Further, the Office Action states that the specification provides no guidance as to how to use the claimed active immunization method for cancer therapy in humans. The Examiner points to several publications that voice doubts as to the potential of cancer vaccines, ignoring any successes. For these reasons, the Examiner states, it would require undue experimentation to use the present invention as claimed. Such a bleak outlook for cancer immunotherapy is unwarranted. Successful instances of cancer immunotherapy have been shown. See for instance, Berd et al. (1986) Cancer Research 46: 2572-2577 (copy attached), cited by Spitler in the reference invoked by the Examiner. Berd et al. describes the induction of successful vaccine-induced anti-tumor immunity in two patients. These patients showed no regression or new tumor formation more than two and a half years after treatment (See in particular, the last two sentences of the Abstract, and summary paragraph, col. 2, page 200).

The predictability in the art as applied to the claimed method based on the observations with DHDK12 and AR42J cells is bolstered by the previous disclosures in the art. See for instance, Watson

et al (1991) Cancer 68(6): 1255-1260, in which the behavior of human colon cancer cell lines LoVo and C146 paralleled that of the rat cell line AR42J in gastrin receptor antagonist studies.

As to specific guidance for treatment with the G17:DT vaccine of the present invention, administration in humans is taught by the present specification at page 4, last two paragraphs, citing co-assigned U.S. Patents 5,023,077 and 5,468,494 with extensive disclosures as to how to make and use the vaccine of the present invention. See for instance, U.S. Patent 5,023,077 columns 4, 5 disclosing preparation of the anti-G17 vaccine, and column 6 for vaccine administration including methods suitable for human subjects.

For all the above-recited reasons, Applicants maintain that claims 1-5 of the present application are adequately enabled by the specification and therefore the rejection of these claims under 5 U.S.C. § 112 first paragraph as allegedly failing to comply with the enablement requirement should be withdrawn.

III. Rejection Under 35 U.S.C. § 112 first paragraph

In the Office Action of November 1, 2005, claims 1-5 were rejected under 35 U.S.C. § 112 first paragraph as allegedly failing to provide a written description in the specification. The Examiner cites University of California v. Eli Lilly & Co. 119 F.3d 1559 (Fed. Cir. 1997) and Enzo Biochem, Inc. v. Gen-Probe Inc. 296 F.3d 1316 (Fed.Cir. 2002) in an attempt to support a position that the specification does not adequately describe a G17Gly-dependent tumor, and therefore furthermore cannot adequately describe a method of using "that product."

The Examiner relies on a quote from the above-identified cases that a generic statement such as "vertebrate insulin cDNA" or "mammalian insulin cDNA" without more, is not an adequate written description of the genus because it does not distinguish the genus from others, except by function..."

However, this argument cannot be applied where, as in the present case, in addition to the generic description, the specification provides an actual example of a species of the genus of G17Gly-dependent tumors. In this case the species of G17Gly-dependent tumor is the DHDK12 tumor cell line.

DHDK12 is a representative member of the class of G17Gly-dependent tumors, as explained above: Briefly, DHDK12 and AR42J cells are rat cell lines shown to have properties representative of human colon cancer cell lines. These include the human LoVo and C146 cell lines which exhibit properties parallel to those of the rat cell line AR42J in gastrin receptor antagonist studies.

Further, G17Gly-dependent tumor cells are recognized by their sensitivity to G17Gly and their response to the reduction or ablation of G17Gly. The present specification teaches that G17Gly-dependent tumors grow in the presence of G17Gly, but exhibit a reduction in growth and/or proliferation as demonstrated by their reduced size and weight after treatment with anti-G17 antibody, either by passive or active immunization.

The claims relating to the genus of G17Gly-dependent tumors is supported by a specific example, and that example, the DHDK12 cell line has been accepted as a representative member of the genus of G17Gly-dependent tumors. Therefore, the arguments advanced in the Office Action do not apply and the specification fully complies with the written description requirement.

Therefore, the rejection of claims 1-5 under 35 U.S.C. § 112 first paragraph for alleged lack of an adequate written description is misapplied and should be withdrawn.

IV. Rejection Under 35 U.S.C. § 112 second paragraph

In the Office Action of November 1, 2005, claims 1-5 were rejected under 35 U.S.C. § 112 second paragraph as allegedly indefinite for the recitation of the term "-dependent" in claims 1-5.

According to the Patent and Trademark Office, claim 1 is unclear as to the meaning of the term "-dependent" in "glycine-extended gastrin-17-dependent gastrointestinal tumors."

Applicants respond that one of ordinary skill in the art after reading the present specification would readily understand that the term "glycine-extended gastrin-17-dependent gastrointestinal tumors" relate to tumors stimulated by glycine-extended gastrin-17 (G17Gly), and that this stimulation results in growth and proliferation, since the present specification discloses that a reduction in G17Gly results in a reduction in tumor size and weight. Therefore, Applicants maintain that the term "-dependent" in the phrase "glycine-extended gastrin-17-dependent gastrointestinal tumors" of claim 1 is clear and definite.

For this reason the rejection of claims 1-5 under 35 U.S.C. § 112 first paragraph for alleged lack of clarity should be withdrawn.

REQUEST FOR RECONSIDERATION

For all the above reasons, Applicants respectfully request reconsideration of the rejection of claims 1-5 of the present application and allowance of these claims.

TIME OF TRANSMITTAL OF AMENDMENT

This Amendment is being filed within three months of the mailing of an Office Action on the merits.

The Commissioner is hereby authorized to charge any fee necessary to maintain pendency of this application, now or during future prosecution of this application to Deposit Account No. 23-1703.

Dated: February 1, 2006

Respectfully submitted,

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Growth-Promoting Effects of Glycine-Extended Progastrin

Catherine Seva, Chris J. Dickinson, Tadataka Yamada*

Peptide α amidation is required to produce some hormones, such as gastrin, from their glycine-extended precursors. This terminal posttranslational processing reaction is thought to be essential for the biological activation of many peptide hormones; only amidated gastrin exerts a physiological effect that results in gastric acid secretion. However, both amidated gastrin and glycine-extended gastrin stimulate proliferation of exocrine pancreatic cell line AR4-2J through selective receptors for the substrate and the product, respectively, of peptide α amidation. Thus, the amidation reaction may function as a determinant of the specific biological actions of products derived from prohormones.

Although characterized as a stimulant of gastric acid secretion (*1*), the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues (*2–5*). As with many peptide hormones, gastrin is synthesized as a precursor molecule that undergoes posttranslational processing to a product amidated on the COOH-terminus, which is presumed to be the sole biologically active form (*6–9*). Posttranslational processing intermediates of gastrin, specifically glycine-extended gastrins (G-Gly), which serve as the substrates for the amidation reaction, are at least four orders of magnitude less potent than gastrin in stimulating gastric acid secretion (*10*). However, G-Gly is stored in brain (*11*) and gut tissues (*12–14*), secreted with amidated gastrin (*15–18*) into the circulation, and achieves concentrations in plasma like those of gastrin. Malignant tissues that express gastrin, such as Zollinger-Ellison tumors and colon cancers (*15, 19, 20*), contain greater concentrations of G-Gly than amidated gastrin. These observations prompted us to examine whether G-Gly functions as a growth factor in a different fashion than its relatively weak effects on the standard receptor, which recognizes gastrin amidated on the COOH-terminus.

Amidated gastrin stimulates proliferation of a tumor-derived pancreatic acinar cell line (AR4-2J) through the classical gastrin-cholecystokinin B (G-CCK$_B$) receptor (*21*). We compared gastrin heptadecapeptide (G17) and its glycine-extended processing intermediate, G17-Gly, for their abilities to stimulate DNA synthesis in the AR4-2J cell line. Both G17 and synthetic rat G17-Gly stimulated [^{3}H]thymidine incorporation in a dose-dependent fashion (Fig. 1), and maximal stimulation was achieved by G17 (142 ± 5% of an unstimulated control; mean ± SE, n = 8) and G17-Gly (140 ± 6%) at a concentration (0.1 nM) similar to that in plasma. A shorter form of glycine-extended gastrin, G5-17–Gly, also stimulated [^{3}H]thymidine incorporation with an efficacy similar to that of G17 (133 ± 2%), although its potency was decreased.

Growth of AR4-2J cells is dependent on cell polyamine content (*22*); amidated gastrin stimulates an increase in the activity of ornithine decarboxylase (ODC) (*23*), the rate-limiting enzyme in polyamine biosynthesis (*24*). G17 and G17-Gly stimulated ODC activity in AR4-2J cells (Fig. 2). The half-maximal effect (EC_{50}) was achieved at a concentration of approximately 0.2 nM for both peptides, and maximal effects were obtained at 1 nM. Difluoromethylornithine (DFMO; 2 mM), an irreversible inhibitor of ODC, completely abolished the stimulatory effects of both G17 and G17-Gly.

The stimulation of AR4-2J cell proliferation by amidated gastrin is mediated through G-CCK$_B$ receptors (*21*). Therefore, we examined the effects of two different selective G-CCK$_B$ receptor antagonists (L365,260 and PD-134308) on proliferation stimulated by G17 and G17-Gly. The antagonists by themselves had no effect on the basal growth of the AR4-2J cells (*25*). L365,260 and PD-134308, used at concentrations that result in full occupation of G-CCK$_B$ receptors (100 nM), completely inhibited the increase in [^{3}H]thymidine incorporation induced by G17 (Fig. 3A). By contrast, neither antagonist decreased the [^{3}H]thymidine uptake stimulated by G17-Gly (Fig. 3B).

These data suggested that G-Gly might

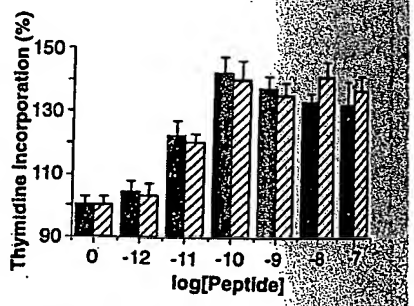

Fig. 1. Effect of G17 (black bars) and G17-Gly (hatched bars) on [^{3}H]thymidine incorporation. AR4-2J cells (*31*) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) were plated, allowed to attach overnight, and then cultured for 24 hours in serum-free medium containing 1 mM unlabeled thymidine. After we washed the cells with serum-free medium, we treated them with increasing concentrations of G17 or G17-Gly. DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into the trichloroacetic acid (TCA)–precipitable material. The [^{3}H]thymidine (0.1 μCi/ml; 18 Ci/mmol) was added during the last hour of the 24-hour treatment period. We then washed the cells with serum-free medium to remove unincorporated [^{3}H]thymidine. DNA was precipitated with 5% TCA at 4°C for 15 min. Precipitates were washed twice with 95% ethanol, dissolved in 1 ml of 0.1 N NaOH, and analyzed in a liquid scintillation counter. Results are expressed as percent of control unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 2596 ± 84 cpm of [^{3}H]thymidine.

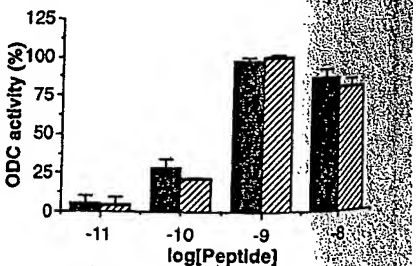

Fig. 2. Effects of G17 (black bars) and G17-Gly (hatched bars) on ODC activity. AR4-2J cells were plated in DMEM supplemented with 10% FBS at 10^5 cells per milliliter, allowed to attach overnight, and then deprived of serum for 18 hours before stimulation. Cells were then incubated with G17 or G17-Gly. ODC activity was measured in cytosolic extracts from AR4-2J cells as described (*23*). Briefly, cells were lysed by freeze-thawing them with liquid N_2 and then centrifuged at 50,000*g* for 25 min. Supernatants were incubated in the presence of pyridoral 5-phosphate (50 μM), L-[^{14}C]ornithine (0.25 μCi), and L-ornithine (0.2 mM). Protein content was measured by the method of Bradford (*32*) with the use of bovine serum albumin as standard. Released [^{14}C]CO_2 was quantitated, and ODC activity was recorded as nanomoles of CO_2 produced per hour per milligram of protein. Results represent the means ± SE of three different experiments, each performed in duplicate, and are expressed as the percent of maximum activity. The basal value of ODC activity was 3.5 ± 0.2 nanomoles of CO_2 produced per hour per milligram of protein, and maximal values after stimulation with G17-Gly and G17 were 5.1 ± 0.1 and 5.2 ± 0.3, respectively.

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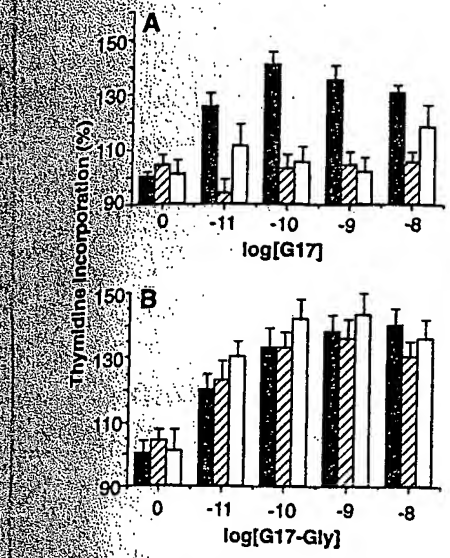

Fig. 3. Effects of the G-CCK$_B$ receptor antagonists L365,260 and PD-134308 on incorporation of [^{3}H]thymidine stimulated by (**A**) G17 or (**B**) G17-Gly in AR4-2J cells. Cells were cultured as described (Fig. 1) and treated for 24 hours with either G17 or G17-Gly alone (black bars), in the presence of L365,260 (100 nM) (hatched bars), or in the presence of PD-134308 (100 nM) (white bars). Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 2708 ± 90 cpm and 2760 ± 150 cpm of [^{3}H]thymidine in (A) and (B), respectively.

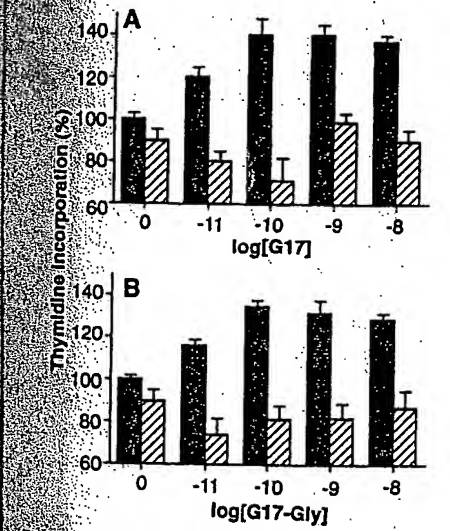

Fig. 4. Effect of octreotide (100 nM) on G17 or G17-Gly–stimulated [^{3}H]thymidine incorporation in AR4-2J cells. Cells were cultured as described (Fig. 1), treated for 24 hours with (**A**) G17 or (**B**) G17-Gly alone (black bars) or in the presence of 100 nM octreotide (hatched bars). Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation (means ± SE of six different experiments, each performed in triplicate). Unstimulated control cells incorporated 3052 ± 110 cpm and 3284 ± 86 cpm of [^{3}H]thymidine in (A) and (B), respectively.

act through a mechanism independent of the G-CCK$_B$ receptors. Somatostatin and its long-acting analogs, such as octreotide (SMS 201–995), inhibit the proliferative action of various growth factors on different cultured cell lines (*26, 27*), including AR4-2J cells (*28*). Octreotide (100 nM) completely inhibited the increase in [^{3}H]thymidine incorporation stimulated by G17 and G17-Gly (Fig. 4). Octreotide reduced the maximal stimulation induced by G17 and G17-Gly (0.1 nM) from 140 ± 8% to 71 ± 11% and from 135 ± 3% to 81 ± 7%, respectively.

If G-Gly has a specific effect on AR4-2J proliferation that is independent of G-CCK$_B$ receptors, it should be possible to demonstrate the presence of G-Gly–selective receptors. The presence of specific G-Gly binding sites on AR4-2J cells was investigated with ^{125}I-labeled human Leu^{15}–G2-17–Gly (*29*). Leu^{15}–G2-17–Gly that was specifically bound increased in a dose-dependent manner (Fig. 5A). The equilibrium dissociation constant (K_d) was 0.45 ± 0.07 nM (n = 7), as determined by Scatchard analysis with the use of the LIGAND program of Munson and Rodbard (*30*), with a binding capacity (B_{max}) of 4.0 ± 2.0 fmol per 10^6 cells (n = 7). By comparison, other K_d and B_{max} values obtained for gastrin binding to AR4-2J cells were 1.1 ± 0.3 nM and 86.9 ± 20.1 fmol per 10^6 cells, respectively (*23*). Competitive binding studies (Fig. 5B) showed that unlabeled Leu^{15}–G2-17–Gly displaced ^{125}I-labeled Leu^{15}–G2-17–Gly bound to AR4-2J cells. However, G17, cholecystokinin octapeptide (CCK), and G-CCK$_B$ receptor antagonists L365,260 and PD-134308 had no effect. The binding of ^{125}I-labeled Leu^{15}–G2-17–Gly to AR4-2J cell mem-

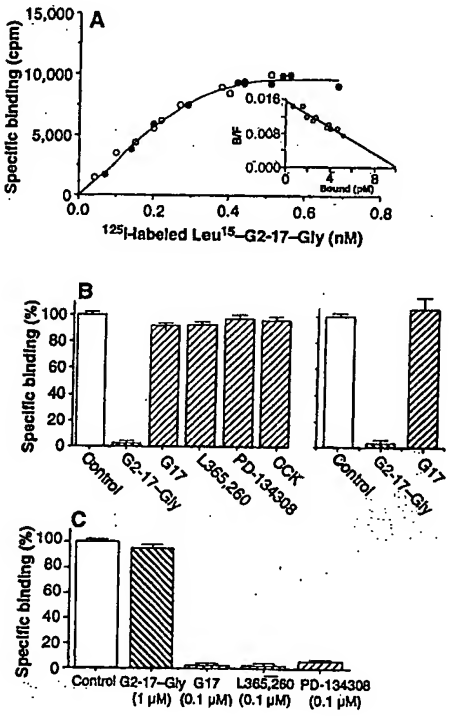

Fig. 5. Binding of ^{125}I-labeled Leu^{15}–G2-17–Gly or ^{125}I-labeled Leu^{15}–G17 to AR4-2J cells and membranes. (**A**) Human Leu^{15}–G2-17–Gly was iodinated with ^{125}I-labeled Na on Tyr^{12} with an adaptation of the chloramine T method and purified by high-pressure liquid chromatography as described (*33*). The specific activity of the label was ~1.5 μCi/pmol. Binding assays were prepared on isolated cells that were detached in phosphate-buffered saline (PBS) containing 0.02% EDTA. Cells (2 × 10^6) were incubated with the indicated concentrations of ^{125}I-labeled Leu^{15}–G2-17–Gly with or without unlabeled Leu^{15}–G2-17–Gly (1 μM) in a Krebs'-*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (Irvine Scientific, Irvine, California) supplemented with 0.5% bovine serum albumin, 0.03% soybean trypsin inhibitor, and 0.05% bacitracin in a total volume of 1 ml at 37°C until equilibrium. Specific binding of ^{125}I-labeled Leu^{15}–G2-17–Gly was calculated per 2 × 10^6 cells as the difference between the total amount of label bound and the amount of label remaining bound in the presence of G17-Gly (1 μM). Nonspecific binding amounted to roughly 20% of the total amount of label binding in the absence of any added G17-Gly. Two saturation binding studies (open and closed circles) are shown; the results are representative of similar data obtained in five additional experiments. (Inset) A Scatchard analysis for one of the binding studies. B, bound; F, free. (**B**) Cells (2 × 10^6; left panel) were incubated with ^{125}I-labeled Leu^{15}–G2-17–Gly without (control) or with 1 μM of unlabeled Leu^{15}–G2-17–Gly; G17; L365,260; PD-134308; or CCK octapeptide, as in (A) at 37°C until equilibrium. For preparation of membranes (right panel), cells were detached and centrifuged at 600*g* for 5 min at 4°C in PBS. The pellet was resuspended in 20 mM tris (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 0.3 mM EGTA, 0.01% soybean trypsin inhibitor, and 0.05% bacitracin. The suspension was sonicated and then centrifuged again for 5 min at 600*g*. The supernatant was recentrifuged at 10,000*g* for 30 min at 4°C. For binding assays, membranes (200 μg) were incubated with ^{125}I-labeled Leu^{15}–G2-17–Gly for 2 hours at 37°C in 300 μl of the same buffer with or without 1 μM G2-17–Gly or G17. Each point represents the mean of three independent experiments, each performed in duplicate. (**C**) Binding studies with iodinated human Leu^{15}-G17 (specific activity of ~1.5 μCi/pmol) were performed on isolated cells that were detached in PBS containing 0.02% EDTA. Cells (2.5 × 10^5) were incubated with 120 pM ^{125}I-labeled Leu^{15}-G17 without (control) or with the indicated concentrations of G17; G2-17–Gly; L365,260; or PD-134308 in a total volume of 0.5 ml at 25°C until equilibrium. Specific binding of ^{125}I-labeled Leu^{15}-G17 was calculated as the difference between the total amount of label bound and the amount of label remaining bound in the presence of 1 μM G17. Each point represents the mean of three independent experiments, each performed in duplicate.

branes was also displaced by unlabeled Leu^{15}-G2-17-Gly, but not by G17 (Fig. 5B). Unlabeled G17 and both G-CCK_B receptor antagonists completely inhibited the binding of ^{125}I-labeled Leu^{15}-G17 to AR4-2J cells, but G2-17-Gly, in concentrations as high as 1 μM, had no effect on ^{125}I-labeled Leu^{15}-G17 binding (Fig. 5C).

Our results indicate that amidated gastrin and its glycine-extended posttranslational processing intermediates induce AR4-2J cell proliferation. In contrast to the difference in the potencies of amidated gastrin and G-Gly in stimulating gastric acid secretion (*11*), the two peptides appear to be equally potent in inducing cell proliferation. Moreover, selective inhibition by L365,260 and PD-134308 of the effect induced by amidated G17, but not by G17-Gly, implies that there are two different receptors that mediate the proliferative actions of the peptides. In view of the observations that both plasma and tissue concentrations of G-Gly are higher than those of amidated gastrin, growth-related receptors for G-Gly may mediate physiological or pathophysiological effects. Our data indicate that the precursor and the product of peptide α amidation may have different biological actions mediated through separate receptors.

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Effect of the Nigrostriatal Dopamine System on Acquired Neural Responses in the Striatum of Behaving Monkeys

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Dysfunction of the nigrostriatal dopamine system results in marked disorders of movement such as occur in Parkinson's disease. Functions of this dopamine-containing projection system were examined in monkeys trained in a classical conditioning task, and the effects of striatal dopamine depletion were tested. Unilateral dopamine loss substantially reduced the acquired sensory responsiveness of striatal neurons monitored electrophysiologically. This effect was ipsilateral and selective, and could be reversed by apomorphine. These results suggest that the primate nigrostriatal system modulates expression of neuronal response plasticity in the striatum during sensorimotor learning.

Understanding the neural mechanisms underlying sensorimotor learning is a cardinal goal of neurobiology. To approach this problem, we investigated neurons of the basal ganglia, central structures in the motor system. We recorded from a clearly identifiable class of neurons in the striatum (the tonically active neurons, or TANs) while monkeys underwent training in a Pavlovian conditioning task. We found that the TANs acquire responsiveness to the sensory conditioning stimuli during behavioral learning (*1*). This systematic learning-dependent plasticity of TANs opened the possibility of determining whether dopamine, a major catecholamine neurotransmitter in the striatum, affects such behaviorally contingent neural plasticity. We therefore recorded from TANs before, during, and after monkeys were trained in behavioral conditioning, and tested the effects of manipulating their dopaminergic inputs (*2*).

Before conditioning, we confirmed that only a small fraction of TANs responded to the clicks used as conditioning stimuli (51 of 305 cells, or 16.7%) (*3*). The TANs were readily identified by their characteristic 2- to 8-Hz spontaneous discharge rates, action potential waveforms, and sparse distribution at 0.5- to 1.0-mm intervals. During training, many TANs became responsive to the conditioned stimuli (Fig. 1). In all, 71.4% of cells (95 of 133) recorded in the caudate nucleus and 52.0% of cells (91 of 175) recorded in the putamen responded to the conditioned stimuli after behavioral conditioning. The responses consisted of a brief pause in tonic firing (Fig. 1D), which began about 60 ms after the conditioned stimulus, lasted about 300 ms, and was often flanked by initial and rebound excitation periods (*2*).

After the conditioned behavior was acquired, we infused 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a dopaminergic neurotoxin, into the caudate-putamen complex of one hemisphere in each monkey (*4*). Unilateral dopamine deficits were evident in the home-cage behavior of the monkeys and were confirmed histologically by immunostaining for tyrosine hydroxylase (TH) after completion of the experiments (*5*). Histology showed dose-related partial (monkey R) to massive (monkey D) loss of TH-like immunoreactivity in the caudate nucleus and putamen, with near total loss near the injection site and graded depletion beyond (Fig. 2A). Postinfusion recordings were made within 5 mm of the injection sites, in the regions of maximum depletion. TH immunostaining was also regionally reduced in the substan-

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Glycine-Extended Gastrin Promotes the Growth of Lung Cancer

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ABSTRACT

The less processed forms of gastrin have recently been shown to act as trophic factors for both normal and malignant colonic cells. Although incompletely processed forms of gastrin such as glycine-extended gastrin and progastrin are also expressed in human lung cancers, the clinical significance of this expression has not been addressed. Consequently, we investigated the effects of overexpression of glycine-extended gastrin in a mouse strain that is prone to developing lung cancer and also examined the expression of incompletely processed gastrins in primary human lung cancers. We found that transgenic overexpression of glycine-extended gastrin in FVB/N mice resulted in a significant increase in the prevalence and growth of bronchoalveolar carcinoma. In addition, a substantial subset of human lung cancers was found to express progastrin and/or glycine-extended gastrin. Overexpression of glycine-extended gastrin by human lung cancers was associated with a significantly decreased survival. Taken together, these results suggest that glycine-extended gastrin may play a role in the growth and progression of some human lung cancers.

INTRODUCTION

Worldwide, lung cancer is the leading cause of cancer-related mortality. In the United States, lung cancer represents the leading cause of death from cancer with an estimated 171,500 new cases in 1998, resulting in 165,500 deaths (1). Over 40,000 new cases are diagnosed each year in the United Kingdom (2). Unfortunately, by the time lung cancer is detected, it is often difficult to treat, resulting in poor (~14% in the United States and ~5% in the United Kingdom) 5-year survival rates (2, 3). A better understanding of the genes involved in the pathogenesis of lung cancer would theoretically lead to novel therapeutic targets in the treatment of lung cancer. The development of lung cancer, as with many other cancers, is a multistage process involving alterations in multiple oncogenes (*e.g.*, K-ras), tumor suppressor genes (*e.g.*, p53), and DNA mismatch repair genes (*e.g.*, MSH1, hMLH1, and MSH2; Refs. 4–7). However, although several of these early genetic changes have been well studied, many of the downstream targets of these signaling pathways have been less thoroughly examined. Some of these downstream targets are growth factors (such as epidermal growth factor, transforming growth factor α, and platelet-derived growth factor) secreted by lung epithelial cells, as well as a variety of neuropeptides such as bombesin/gastrin-releasing peptide (particularly in small cell lung cancer), vasopressin, bradykinin, neurotensin, and gastrin (8).

It has long been recognized that gastrin in its amidated form is an important regulator not only of acid secretion but also mucosal growth, primarily for the fundic mucosa of the stomach (9, 10). Gastrin has been shown to function as a growth factor for the gastrointestinal mucosa *in vivo* and for gastric cancer cell lines *in vitro*. However, it is likely that gastrin has important growth factor functions outside of the gastrointestinal tract. For example, it has previously been shown that gastrin is also expressed in the majority of human lung cancers (11). Although the biological significance of gastrin expression in lung cancer has not yet been fully elucidated, early studies have been intriguing. Zhou *et al.* (12) reported that patients with small cell carcinoma, adenocarcinoma, and squamous cell carcinoma of the lung had elevated levels of gastrin in both their serum as well as in their bronchoalveolar lavage fluid compared with normal controls. In addition, they found that elevated serum gastrin levels correlated with worse prognosis; in addition, gastrin levels fell after curative resection and became elevated again in the setting of new metastases (13). In contrast, other studies have shown no prognostic value for gastrin measurements in the serum or bronchoalveolar lavage fluid in lung cancer patients (14).

Lung cancers, as with most malignancies, lack the neuroendocrine-processing enzymes to make the fully processed amidated form of gastrin. Thus, the vast majority of gastrin synthesized by lung cancers is in the form of the less-processed gastrin intermediates, progastrin, and glycine-extended gastrin (11). Interestingly, it has also been demonstrated that normal lung tissue expresses very low levels of these gastrin-processing intermediates (11). Although in the past it was assumed that incompletely processed gastrins possessed no biological function, more recent studies have shown that these less processed forms of gastrin can act as growth factors for the normal colon (15–17), as well as gastrointestinal cell lines (18, 19). Although additional work is required to characterize relevant receptors, it is clear that progastrin and glycine-extended gastrins have low affinity for the gastrin-CCK-B (CCK-2) receptor.

Furthermore, recent studies have suggested that the less processed forms of gastrin play a role in the pathogenesis of colorectal cancer. Transgenic mice that overexpress progastrin are more prone to colon carcinomas induced by azoxymethane than wild-type controls (20, 21). Mice that have elevated serum levels of glycine-extended gastrin through either infusion or insertion of a transgene are more prone to intestinal polyposis in the APC min mouse model (22, 23), whereas gastrin deficiency generated by either homologous recombination or through an immunogen that raises a gastrin-immunoneutralizing antibody results in a decreased number of polyps in the APC min mouse model (22, 23). Finally, it has been shown that glycine-extended gastrin can promote the invasiveness of human colon cancer cells (24).

Interestingly, we found that transgenic mice overexpressing glycine-extended gastrin (MTI/G-Gly) also spontaneously develop occasional bronchoalveolar carcinomas at 1 year of age (25). These tumors occurred in a genetic background (FVB) that has been reported to show spontaneous development of lung cancer (26). To explore fully the role of gastrin in the pathogenesis of lung cancer, we analyzed further the MTI/G-Gly mice in terms of tumor prevalence, tumor burden, and proliferation rates compared with wild-type FVB

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mice. In addition, we correlated these findings with expression patterns in human lung cancer specimens.

MATERIALS AND METHODS

Generation of MTI/G-Gly Mice. A mouse metallothionein promoter-human glycine-extended gastrin cDNA transgene was used to generate transgenic mice that overexpress glycine-extended gastrin in the FVB background as described previously (25). This transgene resulted in elevated levels of glycine-extended gastrin at the RNA level in all tissues examined (colon, kidney, liver, lung, pancreas, and stomach) and of peptide in the serum, with the predominant circulating form being G34-Gly. One line (7483) was primarily used for this study, although lung cancers were noted in all four MT/G-Gly-transgenic lines that were generated (25).

Animals were housed in microisolator, solid-bottomed polycarbonate cages and fed a commercially prepared pelleted diet and given water *ad libitum*. The mice were all maintained in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility under barrier conditions as virus antibody free mice for the duration of the experiment. The protocol was approved by the animal care committee (Institutional Animal Care and Use Committee) of the University of Massachusetts Medical School.

Analysis of Murine Lung Tumors. Nineteen MTI/G-Gly mice and an equal number of strain-matched (FVB) control mice were sacrificed at 18 months. Twelve MTI-G-Gly mice and 16 FVB mice were sacrificed at 12 months. The lung tissue was then either fixed in 4% paraformaldehyde overnight for eventual embedding with paraffin or in OCT compound and frozen on dry ice. Blocks were sectioned at 5 μm for histological and immunohistochemical analysis and thoroughly examined for lung lesions. A minimum of five sections were examined for each lung. Routine H&E staining was performed for all sacrificed mice, and the number of tumors/lung was quantified according to size and histological phenotype. Ductular hyperplasia/dysplasia is defined as a hyperproliferative state of the bronchial epithelium with or without the presence of dysplastic cells (as evidenced by cytological atypia, loss of nuclear polarity, increased nuclear to cytoplasmic ratio, increased basophilic staining of the nucleus, and increased number of mitoses) with preservation of the underlying bronchial architecture. Adenomas were defined as a circumscribed lesion maintaining glandular architecture lined by dysplastic epithelium that distorts the underlying bronchial architecture. Adenocarcinomas were defined as macroscopically apparent lesions (>5 mm) with invasion of neoplastic cells into surrounding structures, including blood and/or lymphatic vessels.

Patients. Tumor samples were obtained from consenting patients undergoing surgical resection for lung cancer at the Cardiothoracic Centre Liverpool NHS Trust. A diagnosis of squamous carcinoma of the lung was made in 109 patients (mean age, 65.5 years; range, 42.7–87.5 years) and adenocarcinoma of the lung in 143 (mean age, 67.1 years; range, 48.8–82.6 years). This study was undertaken with ethical approval. The specimens formed part of an archival collection.

Immunohistochemistry. For murine tissues, proliferating cell nuclear antigen staining was performed from paraffin-embedded sections from all slides where tumors were found, sectioned at 5 μm, and then deparaffinized in xylene through alcohol. The slides were then placed in 2 M HCl for 1 h at room temperature. The slides were washed in PBS, incubated with horse serum for 30 min, washed again in PBS, and then underwent incubation with a mouse proliferating cell nuclear antigen antibody (working dilution 1:100; DAKOPATTS, Copenhagen, Denmark) and then stained using the Animal Research Kit (DAKOPATTS). The proliferating cell nuclear antigen labeling index was determined as the number of immunopositive cells multiplied by 100 and divided by the total number of cells/high-powered field in either tumors or surrounding normal tissue.

For human studies, samples obtained from paraffin-embedded tissue blocks were arrayed in a 20 × 20 grid (27, 28) and included positive controls of duodenum and stomach (Fig. 3, *A–F*). Sections were processed using antibodies reacting selectively with the main products of the *gastrin* gene. Antibody L382 was raised to a COOH-terminal sequence of human preprogastrin (93–101; *i.e.*, GRRSAEDEN); it reacts with progastrin and its COOH-terminal fragments but not with glycine-extended or amidated gastrins, which do not share this epitope. Antibody L373 was raised to the peptide EEAYGWMDFG corresponding to human preprogastrin 84–93; absorption controls using this peptide, G17, and COOH-terminal progastrin fragments indicate that this antibody reacts with the COOH-terminal Gly-extended gastrins but not progastrin or amidated gastrins. Antibody L425 is directed at the common COOH-terminal amidated sequence shared by the amidated gastrins and cholecystokinin, and absorption controls indicate that it does not react with

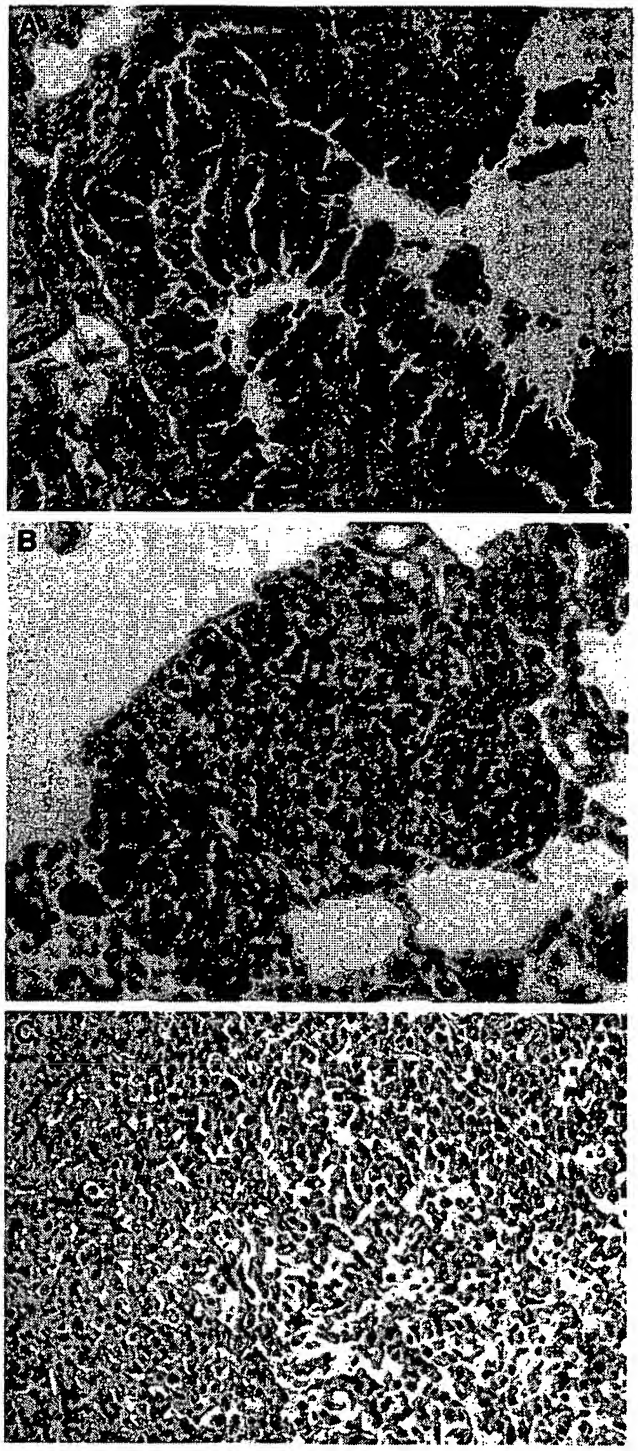

Fig. 1. Lung histopathology in MT1/G-Gly transgenic mice. MTI/G-Gly mice were followed for 12–18 months and then sacrificed, and their lungs were processed for histology. Lesions were scored according to standard pathological criteria (see "Materials and Methods"). This revealed the presence of areas of ductal hyperplasia/dysplasia (magnification, ×200; *A*), alveolar adenoma (magnification, ×200; *B*), and bronchoalveolar carcinoma (magnification, ×200; *C*).

glycine-extended gastrins or progastrin. Endogenous peroxide was blocked with 3% hydrogen peroxide and nonspecific protein blocked with 10% goat serum and 0.25% BSA in 0.05 M Tris-HCl. Primary antibodies were used for 1 h at dilutions of 1:1500 for L382 and 1:1000 for L373 and the Sako LSAB2 HRP labeling kit was used with diaminbenzidine substrate and hematoxylin staining. Sections were screened independently by three observers, including one highly experienced pathologist. The criteria for positive staining in the specimens was staining found in >10% of the tumor cells.

Data Analysis. For the murine studies, statistical analysis was performed using either the χ^2 test or a one-tailed Student's t test. All results are expressed as mean ± SE. For the human studies, all analyses were performed using SPSS 10.0 for Windows. Associations were assessed using the χ^2 and Kaplan Meier for survival analysis. Survival analysis was calculated for the adenocarcinoma specimens on the 63 individuals for whom we had follow-up data.

Table 1 *Progastrin and G-Gly immunostaining in human squamous carcinoma of the lung*

	L382 +	L382 −	Total
L373 +	2	2	4
L373 −	54	51	105
Total	56	53	109

Table 2 *Progastrin and G-Gly immunostaining in human adenocarcinoma of the lung*

	L382 +	L382 −	Total
L373 +	16	10	26
L373 −	48	69	117
Total	64	79	143

RESULTS

MTI/G-Gly Mice Have Significantly Higher Prevalence of Lung Tumors. Transgenic mice overexpressing glycine-extended gastrin (MTI/G-Gly) in both serum and the lung in the FVB mouse strain have previously been described (25). These mice express gastrin at the mRNA level in all tissues examined and have elevated circulating levels of glycine-extended gastrin in the serum (85.0 ± 28.0 pM) compared with FVB mice (<30 pM; Ref. 25). The MTI/G-Gly mice developed ductular hyperplasia/dysplasia (Fig. 1*A*), alveolar adenomas (Fig. 1*B*), and bronchoalveolar carcinoma (Fig. 1*C*), suggesting a stepwise progression toward the development of bronchoalveolar carcinoma similar to that previously published in mice with somatic activation of the k-ras oncogene (29, 30).

At both 12 and 18 months, the MTI-Gly mice had a significantly higher prevalence of bronchoalveolar carcinoma than did the wild-type controls (16.7 *versus* 0%, $P < 0.05$ and 26.3 *versus* 10.5%, $P < 0.05$, respectively; Fig. 2*A*). Of the mice that developed bronchoalveolar carcinoma at 18 months, the MTI-Gly mice also had a higher tumor burden (number of cancers/mouse) when compared with wild-type mice (2.0 ± 0.63 *versus* 1.0 ± 0.0, $P = 0.09$). The tumors of the MTI/G-Gly mice also had a higher proliferation rate as measured by proliferating cell nuclear antigen staining when compared with the tumors found in the FVB mice (15.88 ± 2.37 *versus* 6.83 ± 0.45, $P < 0.05$; Fig. 2*B*).

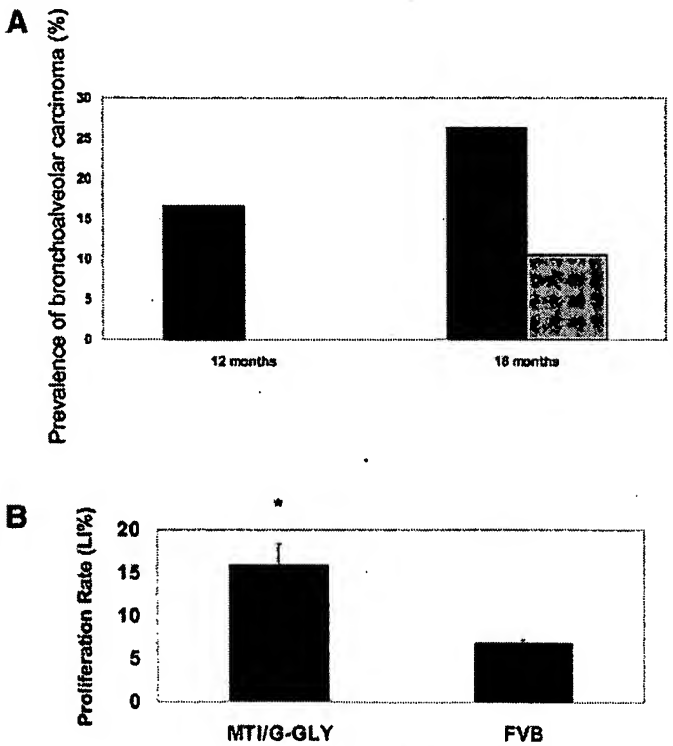

Fig. 2. MTI/G-Gly show increased lung epithelial proliferation and lung cancer prevalence. *A*, prevalence of bronchoalveolar carcinomas in 12- and 18-month-old MTI/G-Gly mice (■) compared with wild-type FVB mice (▨). *B*, proliferation rates as determined from PCNA staining in MTI/G-Gly mice (■) compared with wild-type FVB mice (▨). * indicates a significant increase in proliferation rate in the MTI/G-Gly mice ($P < 0.05$).

Expression of Gly-gastrin and Progastrin in Lung Cancer. Immunostaining was then performed on primary human lung cancers using antibodies against human progastrin, glycine-extended gastrin or amidated gastrin (Tables 1 and 2). Antibodies reacting with progastrin (L382) and Gly-gastrin (L373) revealed consistent staining of some tumors, but antibody to the COOH terminus of amidated gastrins did not show positive staining (Fig. 3, *A–F*). Of 143 samples of adenocarcinoma, 64 were positive for progastrin and 26 for Gly-gastrin. Of the latter, 16 were also progastrin positive but 10 were progastrin negative (Table 2). In the case of squamous carcinoma, 56 of 109 samples were progastrin positive and 4 were Gly-gastrin positive (Table 1). The expression of Gly-extended gastrins in adenocarcinoma was significantly greater than in squamous carcinoma ($P < 0.001$). Interestingly, there was a statistically significant ($P = 0.015$) association between Gly-gastrin expression and decreased survival of adenocarcinoma patients (Fig. 4). The number of squamous carcinoma patients expressing Gly-gastrin was insufficient for an analysis. However, there was no difference in the survival of either adenocarcinoma or squamous carcinoma patients expressing progastrin compared with those not expressing progastrin (Fig. 4).

Progastrin Expression Correlates with More Immature Differentiation Status of the Tumor. One hundred thirty-nine adenocarcinomas with confirmed pathological differentiation grades were analyzed to ascertain whether either progastrin or glycine-extended gastrin expression (or both) correlated with differentiation status of the tumor ($P = 0.018$). Positive staining was found in 24 of 50 poorly differentiated, 34 of 74 moderately differentiated, and 1 of 14 well-differentiated adenocarcinomas. This analysis revealed that adenocarcinomas that stain positively for progastrin were associated with a poorer degree of differentiation when compared with tumors that did not express progastrin (Table 3). Tumors that expressed glycine-extended gastrin did not appear to have a significant shift in differentiation status compared with those that did not express glycine-extended gastrin.

DISCUSSION

In this study, we present evidence that glycine-extended gastrin can contribute to the growth and progression of lung cancer. Expression of glycine-extended gastrin was noted in a substantial subset (*e.g.*,

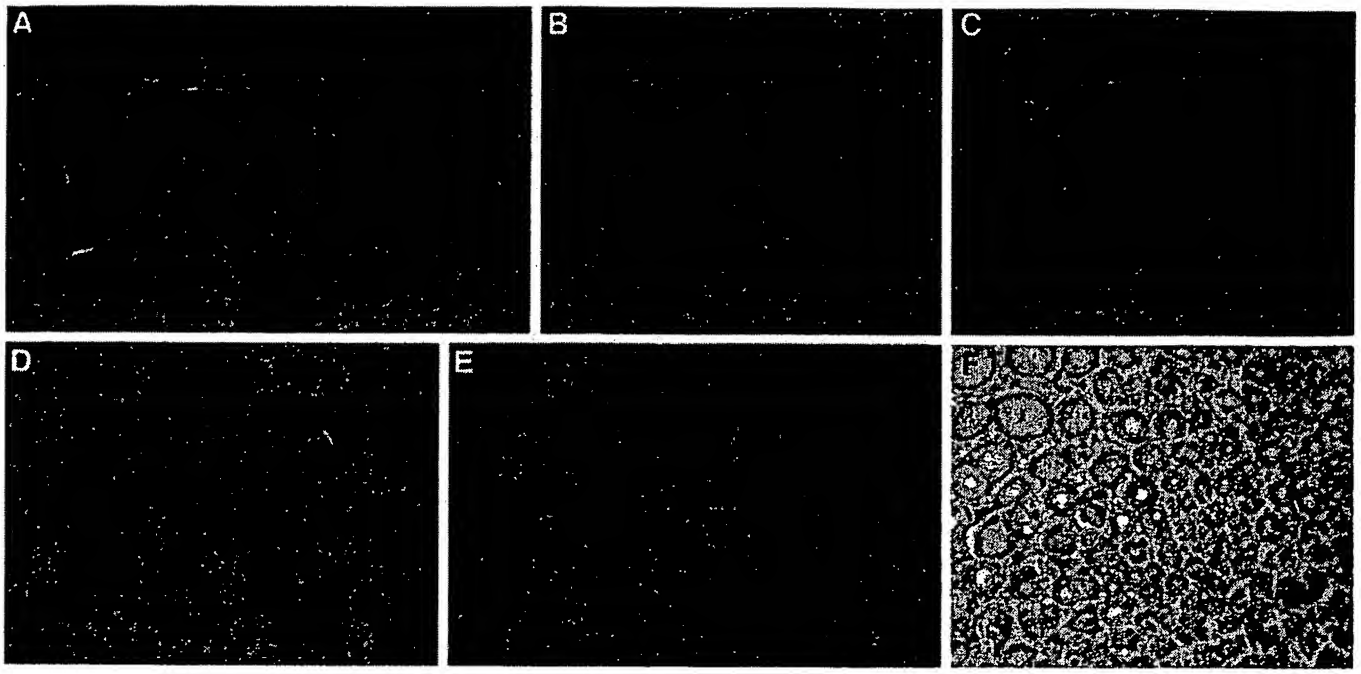

Fig. 3. Expression of gastrin precursor peptides (progastrin and G-Gly) in human lung cancers. A total of 109 human squamous cell carcinomas and 143 human adenocarcinomas of the lung was immunostained to detect progastrin expression (L382) and glycine-extended gastrin (L373). Tumors were determined to be positive if >10% stained positively and to be negative if <10% of cells stained positively. *A* and *B*, positive staining of squamous cell carcinoma with L382. *C*, positive staining of adenocarcimona with L382. *D*, negative staining of adenocarcimona with L373. *E*, positive staining of adenocarcimona with L373. *F*, positive staining of stomach mucosa with L382.

~18%) of human lung adenocarcinomas. Although overexpression of glycine-extended gastrin does not appear to change the differentiation status of the adenocarcinomas, overexpression of glycine-extended gastrin within the carcinoma does correlate with a significantly worse mean survival. Finally, overexpression of glycine-extended gastrin in a mouse strain that is prone to developing bronchoalveolar carcinoma resulted in an increased prevalence and number of bronchoalveolar carcinomas.

Tumors are known to secrete a variety of peptides and growth factors that stimulate in an autocrine fashion their own growth, with the secretion of gastrin-releasing peptide in small cell lung cancer perhaps being the best example. Recent studies have suggested that the less processed forms of gastrin, including glycine-extended gastrin, can act as trophic factors for normal tissue (10, 15, 16, 25), as well as cancer cell lines and tumors (18, 19). Overexpression of both progastrin and glycine-extended gastrin has been shown to induce colonic hyperplasia and accelerate the development of colon cancer (20–22). Although specific receptors have not yet been fully characterized for the incompletely processed gastrins, recent studies from a number of groups have shown that G-Gly can activate mitogen-activated protein kinase and other signaling pathways in gastrointestinal cells (31), whereas progastrin appears to active pp60c-Src kinase (32). In addition, migratory effects of G-Gly have been reported on mouse gastric epithelial (IMGE-5) cells (31). However, although a

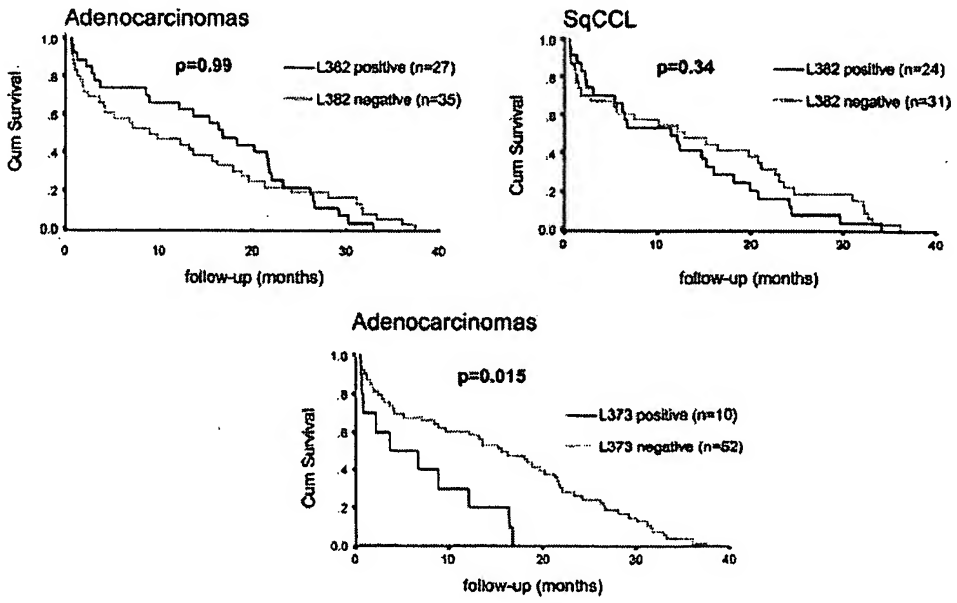

Fig. 4. Expression of G-Gly correlates with decreased survival in patients with lung adenocarcinoma. Kaplan-Meier survival curves were drawn up for progastrin and Gly-gastrin expression in adenocarcinomas and squamous cell of the lung. No difference was found in the survival of either adenocarcinoma or squamous carcinoma patients expressing progastrin (L382) compared with those not expressing progastrin. There was a statistically significant ($P = 0.015$) association between Gly-gastrin expression and decreased survival of adenocarcinoma patients.

Table 3 *Adenocarcinomas that stain positively for progastrin are associated with a poorer degree of differentiation (P = 0.018)*[a]

Adenocarcinoma	PD[b]	MD	WD
Progastrin (+)	24	34	1
G-Gly (+)	6	10	0
G-Gly (−)	18	24	1
Progastrin (−)	26	41	13
G-Gly (+)	2	6	2
G-Gly (−)	24	35	11

[a] Pearson χ^2 P = 0.018 for (+) positive progastrin staining; (−) negative progastrin staining.
[b] PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated.

role for gastrin in gastrointestinal tumorigenesis appears to be well established, relatively less attention has been given to its role in other epithelial tumors.

The expression of gastrin by lung cancer was first recognized in 1989 by Rehfeld *et al.* (11), but there has been little work with respect to the functional significance of this association. With respect to human lung cancer, there have been a number of microarray analyses that have been applied to tumor specimens with the goal of predicting survival or tumor behavior (33). To date, gastrin has not emerged as a major gene of interest from our expression profiling studies (34); however, it is conceivable that this association may have been missed because of posttranscriptional regulation or more likely to the need for subset analysis. In our studies, we were able to confirm the earlier reports that progastrin and, to a lesser extent, glycine-extended gastrin, are expressed in human lung cancers (11), and less than half of tumors were positive for either peptide. In this investigation, adenocarcinomas that expressed progastrin tended to have a more immature differentiation status, whereas only patients with G-Gly-expressing tumors had a change in their mean survival. It is worth pointing out that the poorly differentiated subgroup only constituted 10% of the total number of adenocarcinomas analyzed so that the correlation with differentiation should be treated with caution. In this type of clinical analysis, survival data has to be considered the most powerful clinical indicator, and we therefore place greater weight on the relationship between G-Gly-expressing tumors and clinical outcome.

Although a number of hypergastrinemic mice have previously been described, the MT1/G-Gly mice represent the first reported gastrin-dependent model of lung adenocarcinoma. This propensity for lung cancer development was confirmed in three independent MT1/G-Gly lines and is no doubt related in part to the background genetic strain (FVB/N) that is prone to developing bronchoalveolar carcinoma (26). However, mice in our laboratory that overexpress human progastrin or amidated gastrin (G17) in the FVB background do not appear to have an increased frequency of lung bronchoalveolar carcinomas (data not shown). Although these may suggest specificity of the lung cancer pathway for G-Gly as opposed to G-17 or progastrin, caution is needed in this interpretation because the MT1/G-Gly transgene not only results in increased plasma levels of G-Gly but also is unique in targeting gastrin expression specifically to the lung epithelium, raising the possible need for autocrine action of this growth factor.

The MT1/G-Gly mouse model is similar in some respects to the K-ras^{LA} mouse, another mouse model of lung cancer that shows a more rapid progression to neoplasia (29). In fact, gastrin overexpression in lung adenocarcinoma may be a direct consequence of oncogenic ras mutations. Activating ras mutations have been reported to occur in up to 50% of human adenocarcinomas (35) and in the large majority of mouse lung tumors (36, 37). Furthermore, it has been shown in colon cancer cell lines that activated ras can increase gastrin expression (38), suggesting that the expression of gastrin seen in lung cancers may be secondary to activating ras mutations. Future studies may be needed to address the possible role of gastrin as a downstream mediator of the Ras signaling pathway in tumorigenesis.

In summary, we find that overexpression glycine-extended gastrin appears to increase the frequency of lung carcinomas in a transgenic mouse model. The tumors from glycine-extended gastrin overexpressing mice also have increased proliferation rates. Finally, a substantial minority of human adenocarcinomas also overexpress glycine-extended gastrin, and the overexpression of glycine-extended gastrin appears to be associated with a significantly decreased mean survival. Taken together, this suggests that overexpression of glycine-extended gastrin can play a physiological role in the pathogenesis of lung adenocarcinoma and adds further weight to the therapeutic goal of blocking glycine-extended gastrin in cancer patients.

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Induction of Cell-mediated Immunity to Autologous Melanoma Cells and Regression of Metastases after Treatment with a Melanoma Cell Vaccine Preceded by Cyclophosphamide

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ABSTRACT

There is considerable evidence in animal tumor systems that antitumor immunity is modulated by suppressor T-lymphocytes, and that the cytotoxic drug cyclophosphamide (CY) can abrogate that suppression. We measured the acquisition of delayed-type hypersensitivity (DTH) to autologous melanoma cells in 19 patients with metastatic malignant melanoma. The patients were treated with an autologous melanoma cell vaccine, either given alone, or given 3 days after the administration of CY, 300 mg/m² i.v. The DTH responses of CY-pretreated patients were significantly greater than those of control (vaccine only) patients. Thus, after two vaccine treatments, the median DTH responses (mm induration) were as follows: controls, 4 mm; CY pretreated, 11 mm; $P = 0.034$, Mann-Whitney U test, 2-tailed. Whereas seven of eight CY-pretreated patients developed DTH to autologous melanoma cells of at least 5 mm, only two of seven controls did so ($P = 0.034$, Fisher's exact test). Two patients had significant antitumor responses to treatment with CY plus vaccine, consisting of complete disappearance of skin metastases and a pulmonary nodule in one, and regression of s.c. and liver metastases in the other. Both patients remain free of melanoma after 42 and 33 mo, respectively.

INTRODUCTION

It is well established that immunotherapy can be effective against malignant tumors transplanted into experimental animals (1, 2). However, until recently, it was a commonly held belief that experimental immunotherapy could only be effective when administered before the inoculation of tumor cells, or shortly thereafter, *i.e.*, long before a tumor was palpable (3). The requirement for an extremely small tumor burden seemed to place a serious limitation on the usefulness of immunotherapy for human cancer (4).

It has now become clear that immunotherapy can cause regression of established, grossly evident murine tumors. For example, Berendt and North (5) have achieved immunologically mediated cures of a murine tumor as large as 5 mm in diameter. Such a tumor, having a mass of about 0.4 g, would be approximately equivalent to a 100-g tumor in an adult human patient (calculated on the basis of tumor mass/unit surface area).

This advance in immunotherapy has been made possible by a recognition of the fact that tumor-bearing animals have T-lymphocytes that can specifically suppress the immunological response to the tumor antigens (6–8). Several investigators have now shown that immunotherapy of established murine tumors can only be successful if steps have been taken to deplete or functionally impair these T-suppressor cells (5, 9). This can be accomplished by radical depletion (thymectomy plus whole-body irradiation) and then selective reconstitution of T-lymphocytes (5), or by administration of the cytotoxic drug, CY (10–13).

We hypothesized that pretreatment with CY would enable patients with advanced cancer to develop cell-mediated immunity to tumor-associated antigens to which they would otherwise be unresponsive (12). Moreover, we reasoned that the development of immunity to those antigens could result in regression of metastatic tumor, providing the tumor burden was not too large.

We have tested these hypotheses in patients with metastatic malignant melanoma. We measured the acquisition of DTH to autologous melanoma cells after treatment with a whole cell vaccine, either given alone, or given 3 days after administration of CY. The results confirm that CY pretreatment markedly augments the development of DTH to melanoma-associated antigens, and that the resultant immunity can cause regression of metastatic tumors.

MATERIALS AND METHODS

Design of Study. The study population consisted of 19 patients with surgically incurable, metastatic melanoma. Eligible patients had to have one or more large (>2 cm) metastatic deposits that were easily resectable, *i.e.*, in s.c. tissue or superficial lymph nodes, and residual metastatic deposits that were measurable.

The patients were alternately assigned to one of two groups: (*a*) vaccine alone (control group) or (*b*) vaccine preceded by CY. The clinical characteristics of the patients in the two groups are shown in Tables 1 and 2. The distribution of age, sex, and Karnovksy status in the two groups was similar. Although all of the patients had s.c. and nodal metastases, four in the control group and six in the CY group also had visceral tumor deposits. All but two of the patients had been treated with cytotoxic chemotherapy. One patient in each group had received radiation therapy at least 6 mo prior to entry on this study.

The patients were tested and treated according to the schema shown in Table 3. On Day 0, they were either left untreated or given CY, 300 mg/m², as an i.v. bolus. Three days later, all the patients were given injections of autologous melanoma vaccine. The treatment was repeated every 28 days. Prior to receiving vaccine, and 18 days after each vaccine injection, they were tested for DTH to autologous melanoma cells and to control materials (see below). Patients were continued on the study until there was clear evidence of progression of metastatic disease or until the supply of vaccine was exhausted.

Preparation of Tumor Cells. We used a modification of the method of Peters *et al.* (14). Freshly excised tumor masses were trimmed of skin, fat, and necrotic tissue and minced in cold modified Hanks' medium (Hanks' balanced salt solution plus 1% human AB-positive serum plus 0.1% EDTA plus penicillin plus streptomycin) (M. A. Bioproducts, Bethesda, MD). Cells that were released into the medium by mechanical dissociation were put aside and stored separately. The minced tumor pieces were placed in an enzyme solution, consisting of collagenase, 140 mg, and DNase, 30 mg, in 100 ml of modified Hanks' medium. The collagenase was type I (Sigma Chemical Co., St. Louis, MO) from *Clostridium histolyticum*; the DNase was type I (Sigma).

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[2] The abbreviations used are: CY, cyclophosphamide; DTH, delayed type hypersensitivity; DMSO, dimethyl sulfoxide; BCG, *Bacillus Calmette-Guerin*; i.d., intradermally.

Table 1 *Clinical characteristics of patients*

Patient	Sex	Age	Karnovksy performance status	Metastatic sites	Prior chemotherapy drugs	No. of vaccine treatments given	Survival times after starting vaccine (mo)
CY pretreated							
1	M	58	90	S,[a] LU	DT	7	42+
2	F	61	70	N, LU	DT, meC	10	13
3	M	61	70	S	DT, V, CC, B, CY	2	6
4	F	46	90	S, LU	DT, CC	2	7
5	F	59	70	S, N	DT	3	5
6	F	61	70	S, LU	DT, CC, HN_2	2	6
7	M	48	50	N, LU	DT, CC, VBP	5	4
8	M	56	60	S, LI	DT, Vi, CC, Vin	3	33+
9	M	31	90	S	None	12	32+
Controls							
10	M	59	90	S, N	meC, DT	3	11
11	F	76	90	LU, N	DT, CY	2	10
12	F	54	60	S, N	DT	2	3
13	M	59	90	LI, N	None	2	4
14	M	40	70	S, LI	DT, CC	2	7
15	F	61	60	S, BO, LU	CC, DT	2	6
16	F	48	90	S, N	V, P, DT	3	6
17	F	26	50	S, N	DT	2	6
18	F	52	50	S, N	meC, DT, T, CY, VBP	1	3
19	M	43	70	N	DT	2	1

[a] S, skin, LU, lung, DT, dacarbazine; N, nodal; meC, semustine; V, vinblastine; CC, lomustine; B, bleomycin; VBP, vinblastine-bleomycin-platinum; LI, liver; Vi, vincristine; Vin, vindesine; BO, bone; P, platinum; T, Thiotepa.

Table 2 *Clinical characteristics of patients: summary*

	CY treated	Controls
No. of patients	9	10
Male/female	5/4	4/6
Age (median)	58 (31–61)[a]	54 (26–76)
Visceral metastases	6	4
No. of prior chemotherapies (median)	2 (0–5)	2 (0–7)
Karnovksy status (median)	70 (50–90)	70 (50–90)
Survival time (mo) (median)	7 (4–39+)	6 (1–11)

[a] Numbers in parentheses, range.

Table 3 *Outline of study*

Day of study	Procedure	
	CY group	Control group
−7	Apply skin tests	Apply skin tests
−5	Read skin tests	Read skin tests
0	CY, 300 mg/m² i.v.	No treatment
3	Vaccine 1	Vaccine 1
21	Apply skin tests	Apply skin tests
23	Read skin tests	Read skin tests
28	CY, 300 mg/m² i.v.	No treatment
31	Vaccine 2	Vaccine 2
49	Apply skin tests	Apply skin tests
51	Read skin tests	Read skin tests
56	CY, 300 mg/m² i.v.	No treatment
59	Vaccine 3	Vaccine 3

extracted from bovine pancreas. The dissociation process was carried out in baffled, trypsinizing flasks in a 37°C water bath with constant stirring using magnetic stir bars and an immersible stirrer. After 30 min of dissociation, the enzyme solution containing the cell suspension was removed, and fresh enzyme solution was added. The dissociation process was continued until no visible tumor tissue remained.

The tumor cells were washed twice in modified Hanks' medium, resuspended in freezing medium (RPMI 1640 plus 10% human AB-positive serum plus penicillin plus streptomycin plus 10% DMSO), and frozen in a controlled rate freezer (Union Carbide, Indianapolis, IN) at 1°C/min. They were stored in the liquid phase of liquid nitrogen until needed.

Preparation of Vaccine and Skin Test Material. On the day that a patient was to be skin tested or treated, the cells were thawed, and the DMSO was slowly diluted with modified Hank's medium. Then they were washed in Hanks' balanced salt solution without additives. The cells were irradiated in a cesium irradiator to a dose of 2500 R, washed, and resuspended in plain Hanks' at an appropriate concentration. The volumes were adjusted to a specific concentration of live tumor cells. Cells were identified as tumor cells by size and nuclear configuration. Contamination by dead cells (trypan blue exclusion), leukocytes, and erythrocytes varied from 10–30%. The number of live tumor cells injected did not vary appreciably between skin tests. Bacterial contamination was not observed either by direct microscopy or after short-term tissue culture experiments that were performed as part of an unrelated project.

Mechanically dissociated melanoma cells were obtained by collecting the material released by minced tumor pieces. Although the yields were low (14), sufficient numbers of viable cells were obtained for skin-testing 12 of the patients. Contamination of these preparations by dead cells and leukocytes far exceeded that of enzymatically dissociated cells and was sometimes as high as 90%.

The vaccine consisted of live tumor cells suspended in 0.2 ml of Hanks' solution to which was added BCG, 0.1 ml (approximately 0.8–2.6 × 10^6 viable organisms) (Glaxo, Research Triangle Park, NC). The number of viable cells per vaccine treatment varied from 10–25 × 10^6, depending on the availability of material, but was similar in the CY and control groups [CY, 20 ± 2 (SE); control, 22 ± 1]. For seven of the patients (five CY pretreated, two vaccine only), two or more metastatic deposits were pooled to prepare vaccine. The tumor cell-BCG mixture was injected i.d. in three sites on the upper arms, alternating left and right, excluding arms ipsilateral to an axillary lymph node resection.

DTH Reactions. The patients were skin tested with each of the following materials suspended or diluted in 0.1 ml of Hanks' balanced salt solution: (*a*) 1 × 10^6 melanoma cells; (*b*) 3 × 10^6 autologous peripheral blood mononuclear cells, that had been separated on Ficoll-metrizoate and cryopreserved as described for the melanoma cells; and (*c*) a solution of collagenase and DNase mixed in the same proportions

as the solution used for dissociating tumor cells. The enzyme solution was used at the highest concentration (collagenase, 1.4 ng/ml; DNase, 0.3 ng/ml) that did not produce a primary irritant response in unimmunized subjects. DTH reactions were determined at 48 h by measuring the largest and right-angle diameter of the area of induration and calculating the mean. Biopsy of selected skin test sites showed the mononuclear cell infiltrate that characterizes DTH reactions.

RESULTS

DTH to Autologous Melanoma Cells. Prior to receiving vaccine, most patients did not exhibit significant DTH (*i.e.*, >5-mm induration) to autologous melanoma cells. The median responses were 3 mm in the control group and 2 mm in the CY group (not significantly different). Since it was impossible to determine whether these small skin test responses represented low-level cellular immunity or merely irritant responses, they were treated as "background" responses for statistical purposes. For all subsequent analyses, we subtracted the background response of each patient from his/her response measured after receiving vaccine.

The DTH responses to autologous melanoma cells after administration of vaccine are shown in Fig. 1. The DTH responses of CY-pretreated patients were significantly greater than those of controls (vaccine alone); this difference was apparent when DTH responses were analyzed either after the first or the second vaccine treatment (CY group *versus* control group compared by 2-tailed Mann-Whitney *U* test: after one vaccine treatment, $P < 0.01$; after two vaccine treatments, $P = 0.034$). At the completion of two vaccine treatments, seven of eight CY patients, but only two of seven controls had DTH reactions greater than 5 mm ($P = 0.034$, Fisher's exact test).

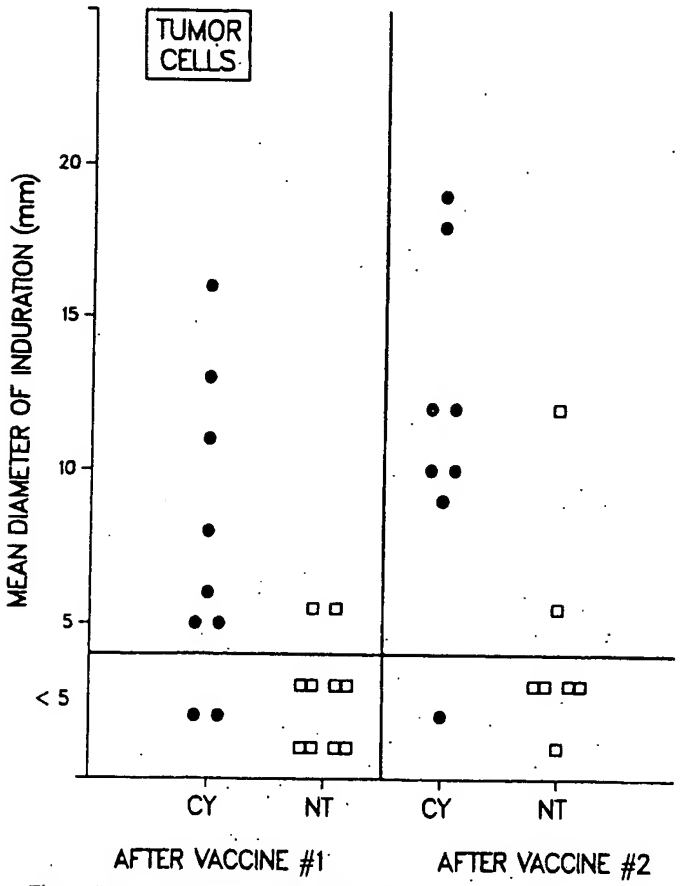

Fig. 1. DTH responses to autologous melanoma cells in patients treated with an autologous melanoma cell vaccine. The patients were either pretreated with CY or given vaccine with no pretreatment (*NT*). Each *point* represents the DTH response (mm, induration) of one patient. The differences between CY and no pretreatment were significant: after Vaccine 1, $P < 0.01$; after Vaccine 2, $P = 0.034$; 2-tailed Mann-Whitney *U* test.

In the CY group, DTH responses to autologous tumor cells were greater after two vaccine treatments than after one treatment. The mean increase in DTH between the first and second vaccine treatments was 4.0 ± 1.0 mm ($P < 0.02$, *t* test for nonindependent samples). Three CY-pretreated patients were skin tested at later time points. In two of these patients, DTH to autologous melanoma cells after three and six vaccine treatments was the same as the DTH response measured after two vaccine treatments. In the third patient DTH continued to increase after the fourth and sixth vaccine treatments.

Antitumor Responses—Vaccine Alone. None of the patients treated with vaccine alone showed any evidence of regression of metastatic tumor. All ten of these patients have died with a median survival time of 6 mo.

Antitumor Responses—CY Pretreated. Six of the CY-pretreated patients had progressive metastatic melanoma and have died with a median survival time of 6 mo. The other three patients are alive at 32+, 33+, and 42+ mo. Despite these long-term survivors, the difference in survival times between the CY- and control group did not reach conventionally defined statistical significance ($P = 0.083$, Kruskal-Wallis analysis).

One CY-pretreated, long-term survivor (32+ mo) had very slowly progressive s.c. metastases while receiving immunotherapy (which was discontinued after 12 mo) but no evidence of tumor regression. The other two CY-pretreated survivors had significant antitumor responses with complete regression of all detectable metastatic melanoma. These two cases are presented in detail below.

Complete Responses to CY plus Vaccine. Patient 1 was a 58-yr-old man who presented with an advanced primary malignant melanoma on the anterior chest. There were dermal metastases of various sizes scattered over the anterior chest and both upper legs. Following chemotherapy with dacarbazine, the lesions on the skin became larger, and a single nodule (15-mm diameter) appeared in the right lung. At that time, treatment was initiated with CY plus vaccine, melanoma cells having been obtained by resection of several of the larger metastatic lesions in the skin. After two vaccine treatments, the lesions on the anterior chest were noted to be smaller. After seven vaccine treatments (approximately 7 mo), the skin metastases had regressed completely (Fig. 2), and the lung nodule had disappeared (Fig. 3). Treatment was discontinued at that time because the supply of vaccine had been exhausted. The patient remained free of disease until 6 mo later, when several small dermal metastases appeared on the right upper leg. These regressed completely after intralesional injection of BCG. The patient remains free of clinically detectable melanoma 42 mo after starting vaccine treatment.

Patient 8 was a 56-yr-old man who had a 2.8-mm-thick primary cutaneous melanoma removed from his left upper arm. Two yr later, he presented with multiple s.c. metastases over his trunk and abdomen, which failed to respond to chemotherapy with dacarbazine, vincristine, lomustine, and vindesine. At the time vaccine treatment was started, he not only had multiple (approximately 20) s.c. metastases, but he also had right upper-quadrant abdominal pain and an enlarged liver by physical examination. A liver scan showed several large defects characteristic of metastatic cancer (Fig. 4). The patient received three courses of CY plus vaccine over a 3-mo period, at which time the supply of vaccine had been exhausted. At the completion of

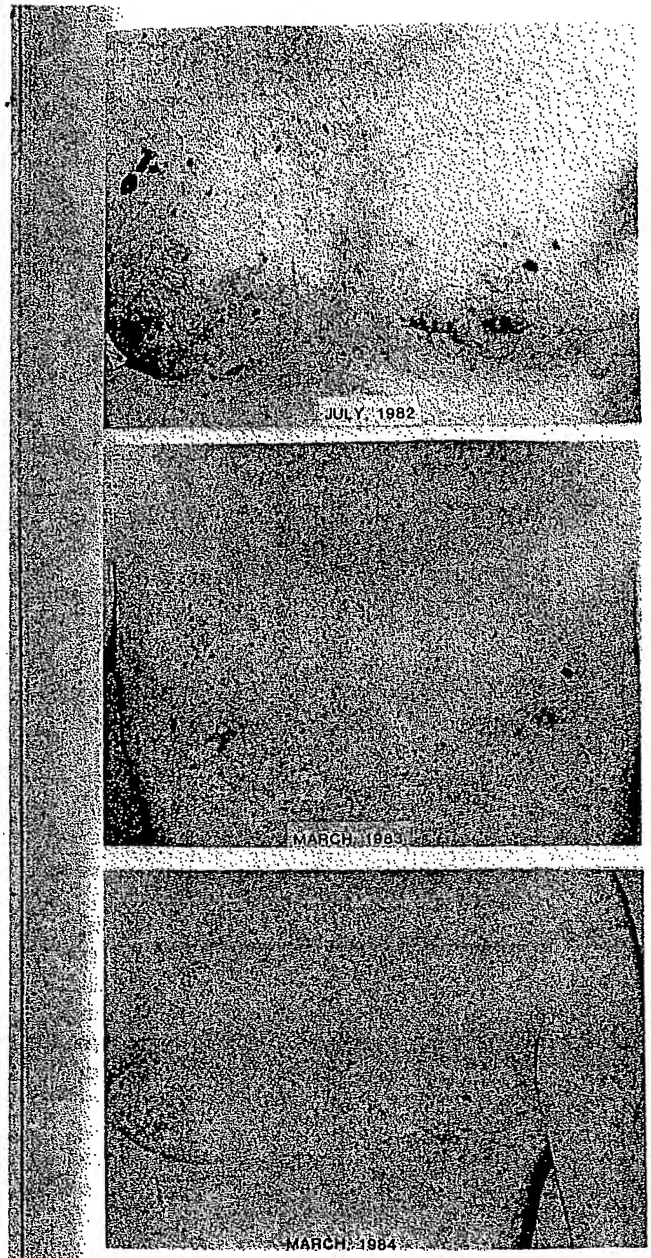

Fig. 2. Regression of skin metastases in Patient 1 after immunotherapy. The large residual pigmented lesion on the left breast is a benign (probably dysplastic) nevus.

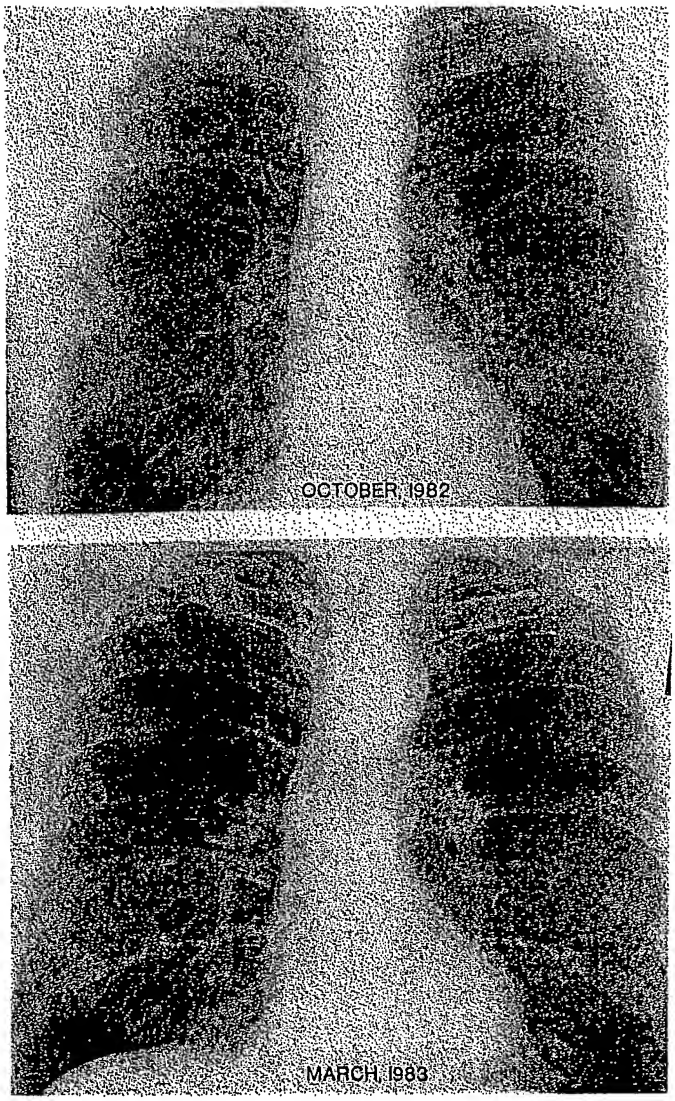

Fig. 3. Chest X-ray of Patient 1 before (*above*) and during (*below*) immunotherapy. Note right upper-lobe nodule (*arrow*) that disappeared during treatment with CY plus vaccine.

treatment, the s.c. metastases were noted to be smaller, and, 3 mo later, they had regressed completely. Another liver scan was performed which showed resolution of all the defects except one in the left lobe (Fig. 4); biopsy of that lesion and examination with melanin-specific stains showed melanin-containing cells with coagulation necrosis. The patient remained well until January 1984, 7 mo after beginning vaccine treatment, when he developed a solitary brain metastasis. This lesion was resected, and the patient was given a course of whole-brain irradiation. Twenty-six mo later (33 mo after starting immunotherapy), he is well and free of clinically evident melanoma.

Toxicity. The only toxicity noted was the local inflammatory response at the vaccine injection site. This consisted of a papule which became ulcerated and drained small amounts of clear fluid with healing by 3–4 wk after the injection. No patients developed fever, chills, or malaise. There were no clinical symptoms suggesting autoimmunity, such as arthralgias. Antinuclear antibody titers were in the normal range for all patients before and after vaccine treatment.

DTH Responses to Other Vaccine-related Antigens. No patients had DTH to autologous peripheral blood mononuclear cells, either before or after vaccine treatment.

Since the vaccine consisted of melanoma cells obtained by dissociation with collagenase and DNase, we tested DTH to those enzymes. No patient reacted to the enzymes before treatment, but after two courses of vaccine, 6 of 9 CY-pretreated patients and 4 of 7 controls had developed significant (>5 mm) DTH responses. For 12 of the patients (5, vaccine alone; 7, CY plus vaccine), we were able to obtain sufficient numbers of melanoma cells by mechanical dissociation, *i.e.*, not contaminated with the enzymes, in sufficient numbers for skin testing. Patients treated with vaccine alone did not develop DTH to autologous, enzyme-free melanoma cells, whereas 6 of 7 CY-pretreated patients did so. Thus CY-pretreatment augmented the development of DTH to melanoma-associated antigens and not just to the contaminating enzymes.

DISCUSSION

Several years ago, we provided the first evidence that CY can augment a human immune response (12). In patients with

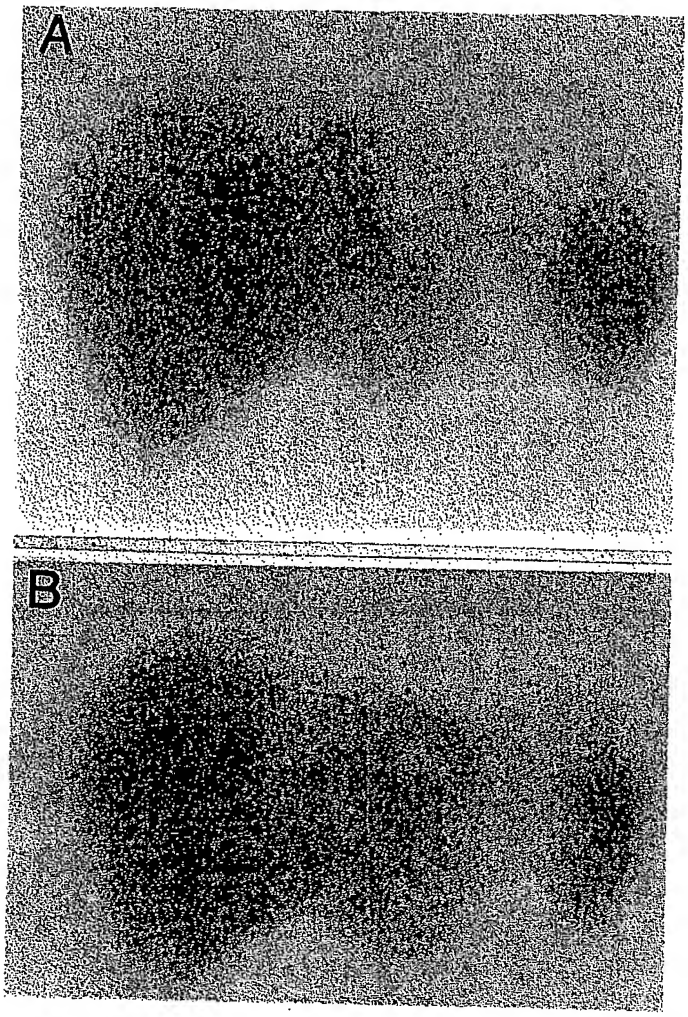

Fig. 4. Regression of liver metastases in Patient 8 after immunotherapy. The pretreatment liver scan (*A*) shows multiple defects throughout the liver. Following CY plus vaccine, the scan (*B*) shows resolution of all the defects except one in the left lobe. Biopsy of that residual lesion was interpreted as necrotic melanoma cells.

advanced cancer, the development of DTH to the primary antigen, keyhole limpet hemocyanin, was markedly augmented by pretreatment with CY, 1000 mg/m² i.v. Subsequently we reported that a lower dose of CY, 300 mg/m², was equally effective in augmenting DTH and, in contrast to the higher dose, augmented the antibody response as well (13). The current study extends our previous observations by showing that CY immunopotentiation may also apply to tumor-associated antigens.

Pretreatment with CY markedly increased the acquisition of DTH to autologous tumor cells in this group of patients with metastatic malignant melanoma. Prior to vaccine administration, there were no significant DTH responses to autologous melanoma cells. This is consistent with previous reports (15) and confirms that evidence of tumor-directed, cell-mediated immunity is rare in patients with advanced cancer. Injection of vaccine alone was not effective in inducing DTH to autologous melanoma cells: only two of the control patients developed small positive reactions. In contrast, injection of vaccine with CY pretreatment 3 days before resulted in the development of DTH to autologous melanoma cells in all but one patient.

In two patients, the acquisition of DTH to autologous melanoma cells was followed by regression of metastatic tumor. This was almost certainly due to CY immunopotentiation: CY alone at this dose has no potential for causing tumor regression in malignant melanoma (13). Both responding patients exhibited complete regression of all cancer and are alive and well 42 and 33 mo, respectively, after beginning vaccine treatment. Following successful immunotherapy, patient 1 developed a new dermal metastasis that regressed after injection with BCG. Patient 8 developed a solitary metastasis in an immunologically privileged site, the brain, that responded to surgery and irradiation. Despite the need for local therapeutic intervention, it seems likely that the current disease-free status of these patients is related to melanoma-directed immunity resulting from the CY plus vaccine administration. However, these therapeutic results must be considered preliminary until confirmed in a larger trial.

Both responding patients had relatively small cutaneous metastases which might have rendered them more favorable candidates for immunotherapy. However, there is strong evidence that visceral metastases also regressed. Patient 1 had a pulmonary nodule that appeared under observation and regressed after treatment. Patient 8 had symptoms and liver scan abnormalities characteristic of liver metastases which resolved after CY plus vaccine administration. A biopsy of a residual hepatic defect showed necrotic melanoma cells.

The failure of the other seven CY-pretreated patients to respond could be explained by the size of their tumor burdens. Although quantitation of human tumor burdens is imprecise, we estimate the tumor burden of Patient 1 at less than 100 g, that of Patient 8 at about 200 g, and those of other patients at 500 g or more.

The specificity of the DTH responses that we observed is an important issue, but one that is difficult to address with our current technological ability. The observation that our patients did not exhibit DTH to autologous blood mononuclear cells implies that neither components of the cell suspension medium (*e.g.*, antibiotics, allogeneic human serum, DMSO) nor cellular changes induced by the cryopreservation process were responsible for inducing DTH. However, it is clear that these patients developed immunity to the enzymes used for tumor dissociation as well as to melanoma-associated antigens. Ideally, one would like to immunize and skin test patients with a purified melanoma-specific antigen. Although such antigens have been isolated (16), they are not yet available in sufficient quantity for clinical use.

There is mounting evidence that active immunotherapy can produce clinically significant antitumor effects. McCune *et al.* (17) reported 4 tumor regressions in 14 patients with renal carcinoma treated with a vaccine made of enzymatically dissociated, intact autologous tumor cells. Tykkä *et al.* (18) have achieved regression of metastases in renal carcinoma patients using a polymerized protein extract as vaccine. Hoover *et al.* (19) have reported encouraging, although preliminary, results in a postsurgical adjuvant trial with colorectal carcinoma.

These reports coupled with our current findings suggest that active immunotherapy could become an important therapeutic modality in human cancer, if it is based on an understanding of the tumor-host relationship. Our demonstration that CY pretreatment augments the development of cell-mediated immunity to autologous melanoma cells suggests that tumor-directed T-suppressor cell activity is an important component of that relationship.

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Inhibitory Effects of the Gastrin Receptor Antagonist (L-365,260) on Gastrointestinal Tumor Cells

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A selective gastrin receptor (GR) antagonist, L-365,260 is bound to the GR on AR42J cells with a potency 7.5-fold less than G17 (50% inhibitory concentration [IC_{50}] G17, 6×10^{-9} mol/l; IC_{50} L365-260, 4.5×10^{-8} mol/l). G17 is mitogenic for AR42J cells, as assessed by ^{75}Se-selenomethionine uptake and L-365,260 at concentrations of 2.5 $\times 10^{-8}$ mol/l and 2.5×10^{-7} mol/l, (55× and 5.5× the dose required to displace 50% of G17, respectively), and reduced optimal G17 stimulated mitogenesis in 75% of experiments. The basal growth of two human colon cancer cell lines, LoVo and C146 was reduced by L-365,260 (2.5×10^{-7} mol/l) after 5 days of treatment to 44% and 64% of the control, respectively. However, inhibition was followed by a rebound of growth to control levels. The growth of AR42J xenografts in nude mice was increased by administration of G17 (10 μg/mouse/d, $P < 0.027$). This increase was blocked by coadministration of oral L-365,260 (5 mg/kg/d, $P < 0.034$). L-365,260 could be an important therapeutic agent in slowing the growth of GR-positive, G17-sensitive gastrointestinal tumors. *Cancer* 68:1255–1260, 1991.

GASTRIN RECEPTORS (GR) were identified first by Brown and Gallagher[1] and have been shown to be present on both human and animal gastrointestinal (GI) mucosa.[2–4] In addition, malignant mucosal cells originating from the gut also have GR.[4,5] The GR appear to have a mitogenic function; gastrin has been shown to be trophic both for normal[6,7] and malignant[8–10] gut mucosal cells.

In a recent study,[11] 67 patients with colon cancer were examined, and 38 (56.7%) had high-affinity, seven (10.4%) had low-affinity, and 22 (32.8%) had no GR. Thus, it would appear that therapy with GR antagonists may be a viable therapeutic option in greater than 50% of colorectal cancer patients.

A benzodiazepine compound recently was described that selectively binds to gastrin and brain cholecystokinin receptors.[12,13] This compound, L-365,260, was shown to bind to GR on a GI tumor cell line and this compared with its corresponding effect on gastrin-stimulated GI tumor mitogenesis.

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Materials and Methods

Established Cell Lines

AR42J is a rat pancreatic acinar tumor cell line.[14] It was shown to possess high-affinity gastrin binding sites.[15] LoVo originated from a human adenocarcinoma of the colon,[16] and C146 was derived in our laboratory from a human colon adenocarcinoma.[17]

All cell lines with the exception of AR42J were grown in Dulbecco's modified Eagle's medium (Flow, Irvine, Scotland) supplemented with 10% fetal calf serum (FCS, Gibco, Paisley, Scotland). AR42J cells were grown in RPMI medium (Flow) containing 10% FCS. All cells were refed three times a week and grown at 37°C in the presence of 5% carbon dioxide.

Gastrin Receptor Antagonist

L-365,260 was provided by Merck, Sharp, and Dohme (West Point, PA) (Fig. 1). For *in vitro* studies, the compound was dissolved in dimethyl sulfoxide (DMSO,

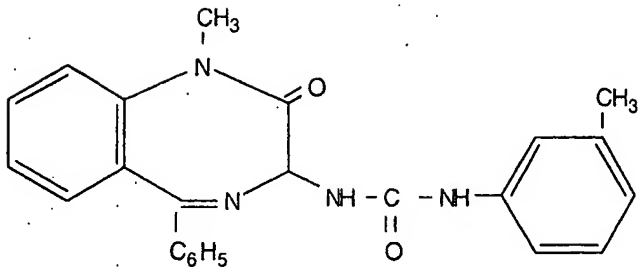


FIG. 1. Chemical definition of L-365, 260 courtesy of Merck, Sharp, and Dohme. Urea, N-(2,3-Dihydro-1-Methyl-2-Oxo-5-Phenyl-1H-1,4-Benzodiazepin-3-YL)-N-(3-Methylphenyl)-, (R)-.

Sigma, Poole, Dorset, UK) and further diluted to the required concentration by infusion into culture medium. For *in vivo* studies, the compound was crushed with a pestle in a mortar into a small volume of phosphate-buffered saline (PBS 0.9%, Oxoid, Basing Stoke, England) containing 0.5% methylcellulose (Sigma).

Gastrin Receptor Binding Studies

For these, the rat pancreatic cell line, AR42J, was used. Initially, a Scatchard analysis was done.[18] Briefly, a semiconfluent monolayer of AR42J cells was harvested with 0.025% ethylenediamine tetraacetic acid (Sigma). The cells were aliquoted into microfuge tubes (Northern Media, Nottingham, United Kingdom) at a cell concentration of 2×10^5 per tube and washed three times with minimal Eagle's medium (MEM, Flow) containing 0.1% bovine serum albumin (BSA, Sigma).

We used ^{125}I-tyrosyl iodinated human gastrin G17 (^{125}I-G17, NEN-Dupont, Stevenage, United Kingdom) with a specific activity 2200 Ci/mmol and concentration of 50 μCi/ml. This was added to the cells at increasing concentrations in the absence or presence of excess unlabeled human G17 (Sigma). All tests were done in either duplicate or triplicate.

The cells were incubated for 1 hour at room temperature before being washed four times in MEM plus 0.1% BSA. Associated radioactivity was counted on a Geiger counter. For analysis of GR on C146 and LoVo, the cells were harvested by scraping off a semiconfluent monolayer, and a single-cell suspension was prepared by vigorous pipetting. Gastrin binding was analyzed at a single dose of ^{125}I-G17 (5×10^{-10} mol/l) in the absence or presence of 5×10^{-6} mol/l unlabeled G17 as described. Specific binding was calculated by subtracting the binding of ^{125}I-G17, in the presence of excess unlabeled G17, from the binding of ^{125}I G17 in the absence of unlabeled G17.

Determination of Peptide Concentrations Inducing a 50% Inhibition of ^{125}I-G17 Binding

AR42J cells were harvested and aliquoted into tubes as described previously. A fixed concentration of ^{125}I-G17 was prepared (final concentration, 5×10^{-10} mol/l) added to increasing concentrations of unlabeled G17 L-365,260 (final concentrations 10^{-6} to 10^{-11} mol/l luted in MEM with 0.1% BSA). These were thoroughly mixed, added to the cells, and incubated as previously described.

Kinetic Measurement of Gastrin Receptor Levels

To compare the GR occupancy abilities of G17 and 365,260, AR42J cells were prepared and incubated with either 5×10^{-6} mol/l G17 or 2.5×10^{-6} mol/l L-365,260 for 1 hour. These were then subsequently pulsed with 5×10^{-10} mol/l ^{125}I-G17 for 1 hour. Parallel aliquots were incubated at room temperature for increasing times before being pulsed with ^{125}I-G17. Specific binding for all samples was calculated.

In Vitro *Mitogenic Assays*

Effect of L-365,260 on gastrin-stimulated mitogenesis. AR42J cells were harvested as described, washed twice in RPMI plus 10% FCS, and plated out in 96-well tissue culture-grade flat-bottomed plates (Flow) with increasing concentrations of G17 (10^{-11} to 10^{-9} mol/l) in the absence or presence of L-365,260 (2.5×10^{-6} mol/l and 2.5×10^{-7} mol/l). Five replicates with drug dilutions were used and DMSO controls were included. Cell proliferation was assessed by ^{75}Se-selenomethionine incorporation as previously described.[19] Results were expressed as a percentage of the untreated control ± standard error of the mean.

Effect of L-365,260 on basal tumor cell growth. Cells were seeded into 96-well flat-bottomed plates at a concentration of 3×10^3 cells/well. The cells were incubated with L-365,260 (2.5×10^{-7} mol/l) for increasing times up to 7 days. On day 4, one half of the plates were refed with L-365,260, and one half were not, to analyze the effect of resupplementation of the GR antagonist on basal cell growth. Refeeding consisted of aspirating the used culture fluid and replenishing with fresh medium containing L-365,260 at the required concentrations. Cell proliferation again was measured by ^{75}Se-selenomethionine incorporation.

In Vivo *Xenograft Studies*

AR42J cells were grown as xenografts in male nude mice (Olac, Bicester, United Kingdom). For this, 10^7 cells were injected subcutaneously into the flanks of the selected animals (25 to 30 g), and after 3 to 5 weeks, tumor growth was evident. The tumors then were aseptically removed, disaggregated mechanically, and redispersed into experimental animals by transplanting 5-μl tubes of tissue subcutaneously.

Therapy consisted of transplanting osmotic mini pumps (14-day, model 2002, Alzet, London, United Kingdom)

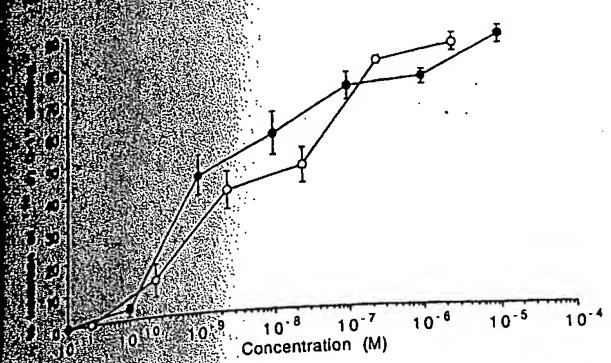


FIG. 2. The ability of increasing concentrations of L-365,260 (○——○) and G17 (●——●) to compete with 5×10^{-10} mol/l of G17 for binding to GR on AR42J cells: G17 (n = 5 expts), L-365,260 (n = 4 expts).

containing either PBS or G17 (10 μg/mouse/d). The PBS or G17 treated groups received either oral PBS or L-365,260 (5 mg/kg/d) in twice daily gavages using a metal canula (1.25 mm diameter, Harvard Apparatus, Kent, United Kingdom). L-365,260 was prepared as previously described and the PBS for control treatment contained 0.5% methylcellulose.

Tumor growth was evident from days 4 to 7. Tumors were measured three times a week with calipers for 3 to 4 weeks by an independent observer. From the perpendicular tumor diameters, the tumor cross-sectional areas were derived. At the end of the experiment, both final tumor weights and the weights of the nude mice without tumors were measured.

Statistical Analysis

All *in vitro* data were analyzed using the Student's *t* test for paired data. The *in vivo* data were assessed using Mann-Whitney U Wilcoxon rank-sum W test with the analysis done by the SPSS/PC+ statistical package (SPSS Inc. Chicago, IL).

Results

Scatchard Analysis

By multiple analyses, AR42J was shown to have a single class of high-affinity binding sites with a dissociation constant of 9×10^{-11} mol/l and a mean number of 5×10^4 binding sites/cell.

Competition Assay Between ^{125}I-G17 and G17 or L365,260 for Binding to AR42J Cells

Both G17 and L-365,260 competitively inhibited the binding of 5×10^{-10} mol/l ^{125}I-G17 to AR42J cells (Fig. 2). Each point on the graph is a mean of five experiments with G17 as the competitor and four experiments for L-365,260. Interexperimental variation is shown.

The 50% inhibitory concentration (IC_{50}) for G17 was 6×10^{-9} mol/l, and for L-365,260, it was 4.5×10^{-8} mol/l, although the two lines were found to superimpose at lower concentrations.

Kinetic Gastrin Receptor Analysis

The length of receptor occupancy observed with both G17 and L-365,260 was compared (Fig. 3). Because the half-life of GR is approximately 3 hours,[20] the cells were treated with G17 (10^{-5} mol/l) and L-365,260 (2.5×10^{-6} mol/l) for 1 hour and assessed for the generation of free gastrin binding sites up to 3 hours later by incubating with ^{125}I-G17. The results are the mean of three replicate experiments expressed as a percentage of the medium control (which measures total binding). The experiment shown is a typical experiment from the series. It was observed that the inhibition of ^{125}I-G17 binding induced by prior incubation with G17 began to reverse after 3 hours to 67% of the medium control and after 4 hours, to 77%. However with L-365,260, the inhibition of binding was greater than G17 (40% after 3 hours and 49% after 4 hours where the difference was significant, $P < 0.05$).

Effect of L-365,260 on the Gastrin-Induced Mitogenesis of AR42J Cells

Figure 4 shows a typical dose–response curve of AR42J cells to human G17. The response is a bell-shaped curve with the optimum G17 concentration between 5×10^{-11} and 5×10^{-10} mol/l. The magnitude of the gastrin response shows interexperimental variation from 170% to 250% of the untreated control. This cannot be controlled by using standardized serum-free medium and cell cultures in the exponential phase of growth.

Table 1 shows the results of four separate experiments showing the effects of G17 (5×10^{-10} mol/l) incubated with and without L-365,260 (2.5×10^{-6} and 2.5×10^{-7} mol/l) on the ^{75}Se-selenomethionine uptake of AR42J cells. L-365,260 significantly lowered the increased label

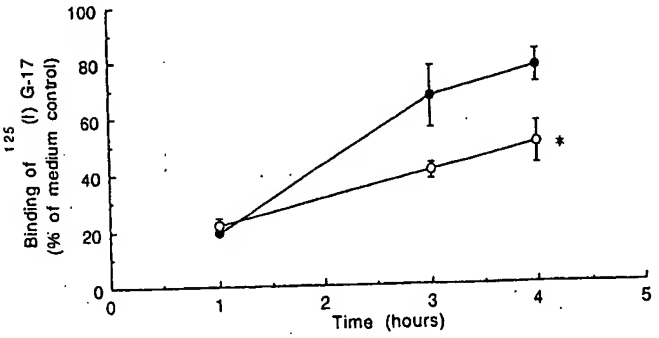


FIG. 3. Binding of ^{125}I-G17 to GR on AR42J cells at increasing time intervals after 1 hour of incubation with G17 (10^{-5} mol/l) (●——●) and L-365,260 (2.5×10^{-6} mol/l) (○——○). Points are the means of triplicate samples. *$P < 0.05$ when compared with untreated controls.

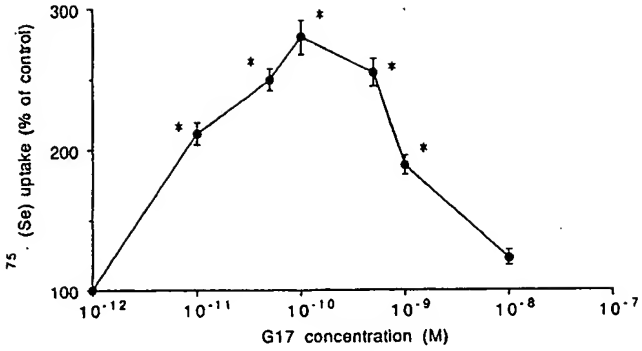


FIG. 4. Effect of G17 on the ^{75}Se-selenomethionine uptake of AR42J cells. *$P < 0.001$ when compared with untreated controls.

uptake in response to gastrin in 75% of experiments. In Experiments 3 and 4, the label uptake in response to gastrin of around 200% of the control was reduced to 120% to 130% ($P < 0.01$ and $P < 0.02$) in the presence of 2.5×10^{-7} mol/l L-365,260. When lower gastrin responses were achieved in Experiments 1 and 2, an insignificant inhibition in the former and a modest inhibition in the latter at 2.5×10^{-7} mol/l L-365,260 ($P < 0.05$) was achieved. A dose response was not achieved; overall 2.5×10^{-6} mol/l L-365,260 did not inhibit the gastrin response more effectively than did 2.5×10^{-7} mol/l.

Effects of L-365,260 on the Basal Growth of Human Colon Cancer Cell Lines

C146 and LoVo cells were not stimulated mitogenically by G17 in *in vitro* culture.[19] However they were shown to possess 2×10^4 and 3×10^3 binding sites/cell, respectively. Recently our group showed gastrin-like immunoreactivity in the culture supernatant of human colorectal tumor cell cultures.[21] If such gastrin binds to external receptors and is necessary for cell growth, L-365,260 should inhibit the basal growth of such cells.

Table 2 shows a typical experiment in which LoVo cells were treated with a single concentration of L-365,260 (2.5×10^{-7} mol/l) with one half of the plates being refed on day 4. On day 5, both the refed and not refed groups had significantly lowered ^{75}Se-selenomethionine uptake (44% and 48% of the control, respectively; $P < 0.001$). However, in both the refed and not refed groups, [illegible] rebounded by day 7 to approach that of the untreated controls.

Table 2 also shows a typical experiment with C146 cells treated with 2.5×10^{-7} mol/l L-365,260 (with the same treatment schedule as for LoVo cells). In the refed cultures on day 5, there was a reduction in the label uptake to 64% of the control value; this was a consistent phenomenon in the other experiments. The cells could not be analyzed on day 7 because the cell cultures were [illegible] confluent, indicating that again L-365,260 did not maintain its inhibitory effect.

In Vivo *Therapeutic Studies With L-365,260*

The following groups of animals were initiated: Group 1, PBS pump (14 day), methylcellulose orally dosed (n = 8); Group 2, PBS pump (14 day), L-365,260 (in methylcellulose) orally dosed at 5 mg/kg/d (n = 9); Group 3, G17 pump (14 day, 10 μg/mouse/d), methylcellulose orally dosed (n = 8); and Group 4, G17 pump (14 day, 10 μg/mouse/d) L365,260 orally dosed at 5 mg/kg/d (n = 8). L-365,260 therapy was administered daily until mice were killed by their excessive tumor burden. Figure 5 shows the mean cross-sectional area (cm^2) of the animals from the four groups on days 10 (Fig. 5, top) and 20 (Fig. 5, bottom). On day 10, there was no difference between the xenograft sizes in any of the four groups. On day 20, mice that received G17 (Group 3) had an enhanced xenograft size that was significant ($P < 0.027$) compared with the PBS-treated controls (Group 1). When L-365,260 and G17 were administered together (Group 4), this enhanced stimulation did not occur (significance from G17-treated group, Group 3, $P < 0.034$). L-365,260 alone (Group 2) had no significant effect on AR42J xenograft growth compared with Group 1.

The weights of the animals were measured when they were killed by their excessive tumor burden or on day 21 (when the experiment was terminated). They were found to be identical in all four experimental groups, indicating that the treatment did not induce any nonspecific toxic effects on the experimental mice (Table 3). The final tumor weights of animals killed either of excessive tumor burden (from day 20 onward) or at the termination of the

TABLE 1. The Effect of L-365,260 Treatment on the Increase in ^{75}Se-Selenomethionine Incorporation Induced by 5×10^{-10} mol/l of G17 on AR42J Cells*

	Mean ^{75}Se-selenomethionine uptake (% of control)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Control (G17 5×10^{-10} mol/l)	168 (4.5)	158 (9.0)	204 (22.0)	210 (20.0)
+ L-365 (2.5×10^{-6} mol/l)	177 (8.0)	131 (14.5)	152 (29.0)	100 (25.0)†
+ L-365 (2.5×10^{-7} mol/l)	146 (22.0)	132 (7.0)†	113 (15.0)‡	120 (20.0)§

* Four separate experiments are shown, and each value is the mean of five replicates ± standard error of the mean.

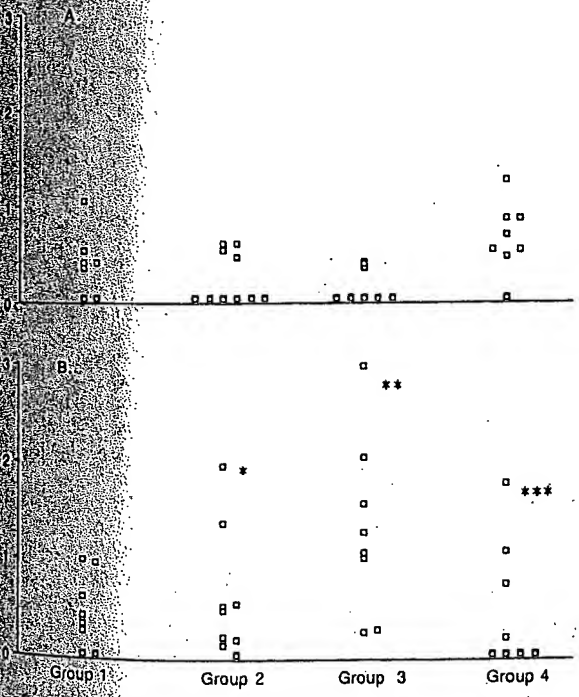
† $P < 0.05$, ‡$P < 0.01$, and §$P < 0.02$ indicate level of significance from untreated control.

TABLE 2. The Effect of 2×10^{-7} mol/l of L-365,260 on the Basal Growth of Two Human Colorectal Cell Lines, LoVo and C146 Cells Over Increasing Times of Incubation, With Resupplementation of L-365,260 at Day 4

Time (days)	Mean ^{75}Se-selenomethionine uptake (% of control ± SEM)	
	LoVo	C146
1	ND	77 (1.0*)
2	98 (4.5)	ND
4	70 (7.0*)	99 (2.0)
5	48 (3.0*)	86 (3.0†)
5 (refed)	44 (8.0*)	64 (4.0*)
7	102 (3.0)	ND
7 (refed)	106.5 (4.0)	ND

SEM: standard error of the mean; ND: not determined.
*$P < 0.001$ and †$P < 0.01$ indicate level of significance from untreated control. Each value is the mean of five replicates ± SEM.

experiment (day 40) reflected the trends observed on day 20. G17 (Group 3) induced an increase in the median tumor size (compared with the PBS controls, Group 1) of 3.3 g to 5.4 g. This was significant ($P < 0.05$). L365,260 alone (Group 2) did not affect the final tumor weights



The growth of AR42J xenografts treated orally with L-365,260 (5 mg/kg/d) in PBS-treated (14-day pump, transplanted at day 0) and G17-treated (14-day pump, 10 μg/mouse/d) nude mice. Group 1: PBS pump (14 day), methylcellulose orally (n = 8); Group 2: PBS pump (14 day), L-365,260 (in methylcellulose) orally (5 mg/kg/d) (n = 9); Group 3: G17 pump (14 day, 10 μg/mouse/d), methylcellulose orally (n = 8); Group 4: G17 pump (14 day, 10 μg/mouse/d), L-365,260 (in methylcellulose) orally (5 mg/kg/d) (n = 8). (Top) Xenograft size at day 10 after tumor transplantation. (Bottom) Xenograft size at day 20 after tumor transplantation. *Nonsignificant from Group 1, **$P < 0.0273$ from Group 1, ***$P < 0.034$ from Group 3, nonsignificant from Group 1 as assessed by Mann–Whitney U Wilcoxon rank-sum W test.

(insignificant in comparison with Group 1). However, when G17 was coadministered with L-365,260 (Group 4), the median weight was smaller compared with when G17 was administered alone (Group 3, $P < 0.01$), but this result was not significantly different from Group 1.

Discussion

Our results show that L-365,260, a GR antagonist, competitively inhibited ^{125}I-G17 binding to GR on the rat pancreatic tumor cell line, AR42J. The IC_{50} for G17 was 6×10^{-9} mol/l and for L-365,260, 4×10^{-8} mol/l. In the competition assay, ^{125}I-G17 did not compete equally with unlabeled G17 on a molar basis. The reason for this is unclear but could be related to the iodinated tyrosine residue influencing receptor binding. Other described antagonists, *e.g.*, proglumide and benzotript, had IC_{50} of 8.6×10^{-3} and 0.4×10^{-3} mol/l, respectively, in competitive assays involving G17 binding to HCT116 colon carcinoma cells.[22] This highlights the superiority of L-365,260 as an antagonist of GR.

Kinetic experiments indicated that L-365,260 was retained on GR longer than G17. However, to maintain receptor occupancy, L-365,260 would have to be readministered as soon as *de novo* receptor synthesis predominated.

The GR present on AR42J are functional; G17 initiates a mitogenic response *in vitro* (as assessed by ^{75}Se-selenomethionine uptake). When this response to G17 was maximal (around 200% of untreated control), L-365,260 induced inhibition. The inhibition did not appear to be dose related; 2.5×10^{-6} mol/l ($55 \times$ the IC_{50}) did not appear to be more effective than 2.5×10^{-7} mol/l ($5.5 \times$ the IC_{50}). Others[23] showed that proglumide (40 nmol/l) reduced the mitogenic effects of gastrin on HT29, a human colon adenocarcinoma cell line.

L-365,260 reduced the basal growth of LoVo and C146 cells. The effect was delayed until day 5 of treatment, and C146 cells required resupplementation with L-365,260 to achieve inhibition. With LoVo cells, inhibition was followed by a rebound effect, indicating a cytostatic effect. Experiments in serum-free medium (with L-365,260 replenished at more frequent intervals) are underway.

TABLE 3. The Effect of Oral L-365,260 (5 mg/kg/d) on the Final Median Tumor Weights in Nude Mice Transplanted With Either 14-Day PBS or G17 (10 μg/mouse/d)-Containing Osmotic Mini Pumps

Group	Median tumor weight (g)
1. PBS pump, oral PBS	3.3
2. PBS pump, oral L-365,260	3.7
3. G17 pumps, oral PBS	5.4*
4. G17 pumps, oral L-365,260	3.2†

PBS: phosphate-buffered saline.
* $P < 0.05$ when compared with Group 1.
† $P < 0.01$ when compared with Group 3, but nonsignificant from Group 1.

Proglumide and benzotript were found to have antiproliferative effects on human colon cancer cell lines in both serum-supplemented and serum-free medium.[22] On removal of the cells from the antagonists, an acceleration to the control growth rate was found, again indicating the cytostatic effect of such antagonists.

The inhibitory effect of L-365,260 on the basal growth of these two human colon adenocarcinomas may be related to the fact that gastrin-like immunoreactivity associated with human GI tumors can be demonstrated[21] and that this tumor-associated gastrin may be involved in growth regulation. The possibility that gastrin could function as an autocrine growth factor was suggested.[22,24] Basal growth of a colon cancer cell line growing in serum-free medium was found to be inhibited by an antigastrin polyclonal antibody, and the inhibition could be reversed by preabsorbing the antiserum with gastrin.[22]

If the cytostatic effects of GR antagonists are reproduced in clinical trials, then such compounds should induce a slowing in growth rather than tumor regression and would require continuous administration. Our *in vivo* experiment with L-365,260 showed that G17 administered continuously by an osmotic mini pump (10 μg/mouse/d) induced enhanced growth of AR42J xenografts after 20 days ($P < 0.027$). Administered orally (5 mg/kg/d), it had no effect on the basal growth of AR42J, but when coadministered with G17, it suppressed the G17-stimulated increase in xenograft growth ($P < 0.034$). Others[25] found that proglumide reduced G17 stimulated growth of the mouse colon cancer xenograft (MC26) but had no effect on basal growth.

In future experiments, we will analyze the *in vivo* effect of L-365,260 on G17-stimulated growth in a gastrin-responsive xenograft system involving the human gastric adenocarcinoma cell line, MKN45.[26] Because GI tumors appear to have GR,[5,11] therapy with potent GR antagonists such as L-365,260 may be effective in reducing the growth of hormone-dependent GI tumors.

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